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IMPAIRED GLUCOSE METABOLISM IN THE ABSENCE OF SKELETAL MUSCLE BRAIN AND MUSCLE ARNT-LIKE-PROTEIN 1 (*BMAL1*)

Brianna D. Harfmann
University of Kentucky, harfmannbd@gmail.com

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Brianna D. Harfmann, Student

Dr. Karyn A. Esser, Major Professor

Dr. Bret Smith, Director of Graduate Studies

IMPAIRED GLUCOSE METABOLISM IN THE ABSENCE OF SKELETAL MUSCLE BRAIN AND MUSCLE ARNT-LIKE-PROTEIN 1 (*BMAL1*).

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
Brianna Dawn Harfmann
Lexington, Kentucky

Director: Dr. Karyn Esser, Professor of Physiology
Lexington, Kentucky
2015

ABSTRACT OF DISSERTATION

IMPAIRED GLUCOSE METABOLISM IN THE ABSENCE OF SKELETAL MUSCLE BRAIN AND MUSCLE ARNT-LIKE-PROTEIN 1 (*BMAL1*)

Metabolism is a critical physiological function that works to generate energy for cells, store substrates and maintain homoeostasis. Alterations in normal metabolism can have a severe effect on physiology, leading to metabolic disease. Skeletal muscle is a key metabolic tissue, taking up ~80% of postprandial glucose. Therefore it contributes considerably to glucose metabolism: glucose uptake, oxidation and homeostasis. To address the role of the skeletal muscle clock in insulin sensitivity and glucose tolerance, our lab generated an inducible skeletal muscle specific *Bmal1*$^{-/-}$ mouse (iMS*Bmal1*$^{-/-}$). 5 weeks post-recombination we observed impairment in both insulin- and AICAR-stimulated skeletal muscle glucose uptake. RT-PCR and western blot analysis demonstrated a significant decrease in mRNA expression and protein content of the skeletal muscle glucose transporter, *Glut4*. Glucose uptake may be affected by glucose utilization so we examined aspects of glycolysis in the skeletal muscle. Both mRNA expression and activity of rate limiting enzymes hexokinase 2 (*Hk2*) and phosphofructokinase 1 (*Pfk1*) were significantly reduced. Additionally, metabolomics illustrated a reduction in metabolites of the glycolytic pathway further supporting a decrease in glycolytic flux. These changes in skeletal muscle glucose metabolism led to altered overall body metabolic health. iMS*Bmal1*$^{-/-}$ mice presented with glucose intolerance and non-fasting hyperglycemia. Furthermore, changes in body composition were seen from 5-12 weeks post-recombination. These data propose a critical role for skeletal muscle *Bmal1* in both skeletal muscle glucose metabolism and overall body metabolic health. The presented findings also illuminate skeletal muscle *Bmal1* and circadian rhythms as potential targets for metabolic disease.

KEYWORDS: Circadian rhythms, glucose metabolism, skeletal muscle, glucose uptake, glycolysis.

Brianna Harfmann .
Student's Signature

October 7, 2015
Date

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By

Brianna D. Harfmann

Karyn A. Esser .
Director of Dissertation

Bret Smith .
Director of Graduate Studies

October 7, 2015 .
Date

Dedication

This work is dedicated to my family and friends who supported me through all the highs and lows of my Ph.D.

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CHAPTER 1: Introduction and Background

1.1 General Introduction

The importance of skeletal muscle to health is often underestimated, yet skeletal muscle is the most abundant tissue in the human body, comprising approximately 45% of total body mass (Goodpaster et al., 2000; Hoppeler and Fluck, 2002). Skeletal muscle tissue is an intricate network of over 600 individual muscles that have different fiber type compositions, metabolic capacities and mechanical functions (Poole, 1986). As a whole, skeletal muscle tissue is critical for systemic health and quality of life. It is well understood that skeletal muscle functions to produce force and enable locomotion, but skeletal muscle is involved in a number of integral processes that are frequently forgotten. Skeletal muscle serves as a principal reservoir for amino acids in the absence of nutrient intake, thereby allowing the maintenance of protein synthesis in other tissues. This supply of amino acids also serves as a pool of precursors for hepatic gluconeogenesis, which is significant in sustaining blood glucose levels in the fasting state (Ripperger et al., 1995). Not only does skeletal muscle serve as a reserve of amino acids, but it also acts as a depot for glucose in the postprandial state. As much as 80% of postprandial glucose is taken up by skeletal muscle (Defronzo et al., 1981; Ferrannini et al., 1988). Consequently, skeletal muscle is vital for systemic level glucose homeostasis.

Changes in muscle composition and function are strongly correlated with disease development. One the most common conditions in the United States is metabolic disease. This category of disease includes but is not limited to diabetes, obesity,

insulin resistance and impaired glucose tolerance. In the U.S., as much as 22% of the population has diabetes mellitus, 17% exhibits impaired glucose tolerance, 32% are obese and approximately 35% meet criteria for metabolic syndrome (Golden et al., 2009). Metabolic disorders are a major health issue and have an unmistakable association with impaired muscle metabolism (Nuutila et al., 1993; Petersen et al., 2007; DeFronzo and Tripathy, 2009; Mitrou et al., 2011; Preisler et al., 2015). Two critical metabolic functions of skeletal muscle are glucose uptake and glucose oxidation. These functions both help maintain glucose homeostasis and provide energy for skeletal muscle cells. It is clear that changes in skeletal muscle metabolic function, which often manifests as altered glucose uptake and substrate oxidation, affect human health. Recently however, a new skeletal muscle property has been implicated in the regulation of skeletal muscle metabolism and the conservation of metabolic health. Skeletal muscle, like virtually every cell in the body has circadian rhythms. Recent studies have begun to demonstrate that disruptions in circadian rhythms may play a crucial role skeletal muscle metabolic dysfunction and metabolic disease development (Yoo et al., 2004; Andrews et al., 2010; Dyar et al., 2014).

Although it is accepted that skeletal muscle cells have their own circadian rhythms, the concept of skeletal muscle circadian rhythms is still quite new. Even more recent is the idea that skeletal muscle circadian rhythms contribute to skeletal muscle metabolism, but very little is yet known. This dissertation aims to further investigate the role of skeletal muscle circadian rhythms in one of the more critical metabolic functions of skeletal muscle, glucose metabolism. In addition, it explores

the idea that exclusive disruption of skeletal muscle rhythms, and therefore skeletal muscle metabolism, will have an effect on overall metabolic health.

1.2 Circadian rhythms, skeletal muscle and metabolism

1.2.1 Circadian rhythms in skeletal muscle

As previously stated, skeletal muscle, similar to almost every cell of the body, has circadian rhythms. These rhythms are generated by a transcriptional-translational feedback loop known as the molecular clock and this mechanism has been reviewed in more complete detail elsewhere (Shearman et al., 2000; Buhr and Takahashi, 2013; Robinson and Reddy, 2014). At a basic level, *Clock, Bmal1, Cry1/2* and *Per1/2* are all core molecular clock components (Kume et al., 1999; Shearman et al., 2000). *Bmal1* and *Clock* make up the positive limb of the molecular clock. They are bHLH transcription factors, which heterodimerize in the nucleus and transactivate *Per* and *Cry* family genes by binding E-box elements in their regulatory regions. PER and CRY then accumulate, multimerize, and translocate to the nucleus where they inhibit BMAL1:CLOCK activity, thereby repressing their own expression. The accumulation of PER and CRY protein is also tightly controlled through phosphorylation and degradation via E3 ubiquitin ligases and the proteasome system so that inhibition of BMAL1:CLOCK activity is lifted (Gallego and Virshup, 2007; Yoo et al., 2013) (Figure 1.1). Proper timing of the

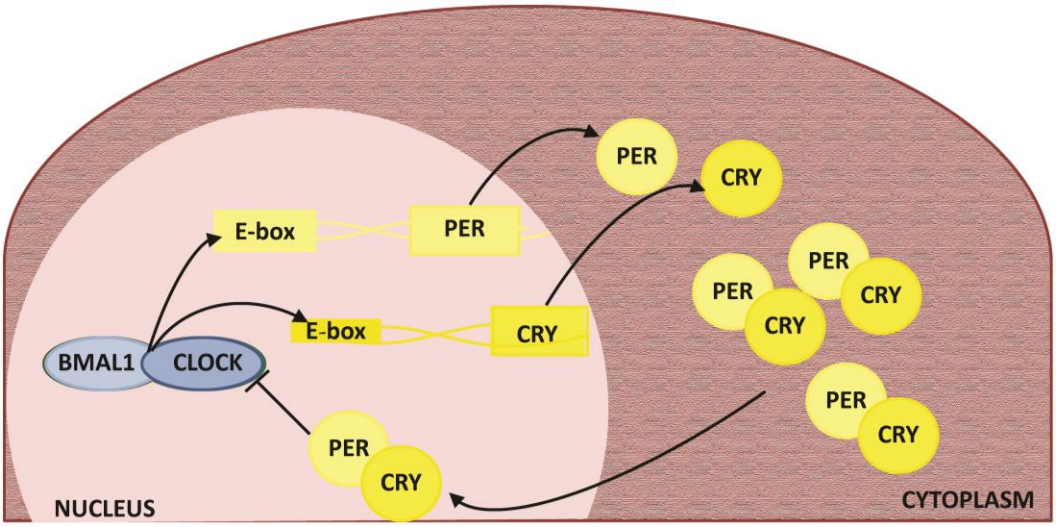


Figure 1.1. Diagram of the skeletal muscle molecular clock. CLOCK and BMAL1 heterodimerize and bind to e-box sequences in the promoter of the period (*Per*) and Cryptochrome (*Cry*) genes, promoting *Per* and *Cry* transcription. After PER and CRY are translated in the cytoplasm, they heterodimerize and are translocated to the nucleus where they inhibit the activity of BMAL1:CLOCK, thereby inhibiting their own transcription. In this general manner an oscillation of ~24 hours is generated.

molecular clock mechanism requires transcription, translation and important rate modifying post-translational steps, thus, presenting many sites through which information from environmental cues and physiological function can support or modify the clock.

Aside from a timekeeping role, the clock modulates the transcription of a large number of genes within the cell (clock-controlled genes [CCGs]); some of these are regulated directly by the binding of the core clock transcription factors Bmal1 and/or Clock to their promoters. To date, the identities of the direct clock controlled genes in a specific tissue, like skeletal muscle, have not been defined, but circadian transcriptome results suggest that they often encode transcription factors (e.g. *MyoD1*, *Pgc1α*) or proteins that control rate-limiting steps in cell physiology (e.g. *Pdk4*, *Dbp*) (Figure 1.2). Tissue specific detailed reviews of the molecular clock mechanism and CCG's within individual tissues are available in several recent reviews by other groups

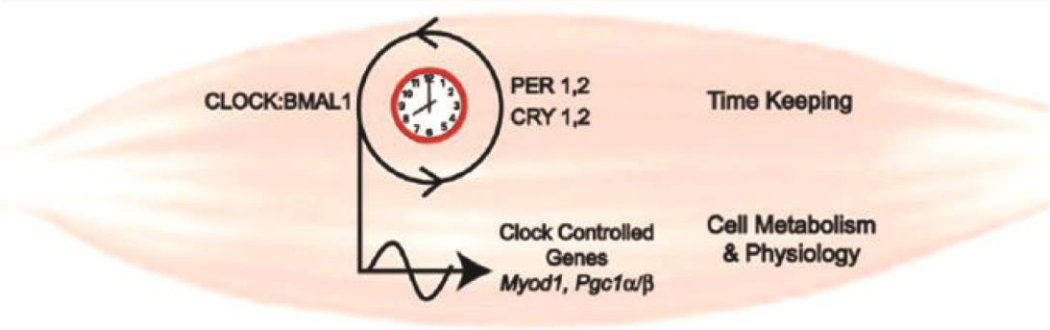


Figure 1.2. Simplified cartoon highlighting the function of the molecular clock as a time-keeping mechanism as well as modulating expression of genes important for skeletal muscle function (in particular metabolism).

(Panda et al., 2002; Storch et al., 2002; Kornmann et al., 2007; Nakahata et al., 2008; Lee et al., 2013; Shostak et al., 2013). The most direct evidence for the contribution of BMAL1:CLOCK regulation of gene expression outside of the timekeeping function comes from chromatin immunoprecipitation studies followed by DNA sequencing. These studies, performed in liver, determined that the BMAL1:CLOCK transcription factors bind to over 2000 sites across the chromatin with up to 85% of the sites being associated with actively expressed genes. Gene ontology analysis showed that the BMAL1:CLOCK targeted expressed genes are highly enriched for metabolic, cancer and insulin signaling pathways in liver (Rey et al., 2011; Koike et al., 2012). Chromatin immunoprecipitation studies have not yet been performed in skeletal muscle, but the results from liver highlight the breadth of transcriptional regulation by the molecular clock factors BMAL1 and CLOCK.

Although more thorough investigations of molecular clock targets are needed in skeletal muscle, there have been studies identifying circadian-expressed genes using expression profiling. The first paper to define circadian gene expression in skeletal muscle was published in 2007 and identified 215 circadian genes (McCarthy et al., 2007).

However, increased sampling frequency, with tissues collected every two hours for 48 hours, and continued development of analysis tools for circadian gene expression studies has expanded this list to greater than 2300 genes (Pizarro et al., 2013). A large portion of these genes are involved in metabolism, transcription and signaling in muscle (McCarthy et al., 2007). The function of the molecular clock in skeletal muscle is only starting to be uncovered. Our lab has quite recently conducted a study analyzing publicly available, high-resolution microarray data in the skeletal muscle of mice. A total of 1,628 mRNAs were classified as circadian mRNAs; genes that showed an approximate 24 hour rhythmicity. Gene ontology was used to look for processes that were enriched in skeletal muscle circadian mRNAs. Of the identified 1,628 skeletal muscle circadian genes, 62% were enriched for metabolic processes. This provides strong evidence for a function of the skeletal muscle molecular clock in regulating skeletal muscle metabolism. Of particular relevance to this dissertation are the results obtained from investigating changes in gene expression when the skeletal muscle molecular clock is impaired. To look at this, we examined the skeletal muscle of mice that had a skeletal muscle specific induced knock out of the core molecular clock gene, *Bmal1* (iMS*Bmal1*$^{-/-}$ mice). The skeletal muscle process most affected by loss of skeletal muscle *Bmal1* was carbohydrate metabolism (Hodge et al., 2015b). This data suggests that one of the major roles of skeletal muscle *Bmal1* and circadian rhythms is to regulate skeletal muscle carbohydrate metabolism.

1.2.2 Entrainment of skeletal muscle circadian rhythms

Circadian rhythms are oscillations over a period of approximately 24 hours, and in skeletal muscle these rhythms include oscillations in transcription, myogenic capacity and notably metabolism (Andrews et al., 2010; Chatterjee et al., 2011; Zhang et al., 2012). This oscillation of physiological processes is believed to be beneficial as it allows an organism to anticipate changes in environmental cues. These rhythms run in the absence of any external environmental cues, but a fundamental property of circadian rhythms is the ability to be entrained. The molecular clock has a phase, defined as the time relative to a specific point of the circadian cycle. For example, phase of the molecular clock may be determined by the time of peak *Bmal1* expression. Entrainment occurs when the phase of the molecular clock is reset or modulated to be aligned with the timing of an environmental cue, such as light (Roenneberg et al., 2003). The skeletal muscle molecular clock can be entrained by cues such a light, time of feeding and activity. In the case of light, the skeletal muscle clock is entrained in an indirect manner through the central clock in the suprachiasmatic nucleus (SCN). Light is transmitted via the retinohypothalamic tract from the retina to the SCN. Light evokes signaling in the SCN, through elements such as cyclic-AMP, that then modulate the molecular clock to affect the peak/phase of molecular clock oscillations (Gooley et al., 2001; Panda et al., 2002b; Lee et al., 2010; An et al., 2011). The SCN molecular clock communicates with other tissues, such as the skeletal muscle, using neurohumoral and temperature signals (Balsalobre et al., 2000; Brown et al., 2002; Abraham et al., 2010; Saini et al., 2012). It is in this indirect manner that the skeletal muscle clock is modulated by light cues.

Time of feeding also serves as an entrainment cue. Studies of time restricted feeding in mice have demonstrated a phase shift in core molecular clock genes in liver

and adipose tissue (Hara et al., 2001; Stokkan et al., 2001; Zvonic et al., 2006). In liver, this was shown to be independent of SCN input, since time restricted feeding prevented the shift of the clock genes when the mice were exposed to a 7-hour light:dark cycle advance (Hara et al., 2001). Although research on time of feeding and skeletal muscle is limited, time of feeding has been shown to entrain skeletal muscle circadian rhythms. A study from our lab demonstrated this using PER2:LUC mice. These mice were developed by Yoo et. al. and have luciferase cDNA knocked into the *Per2* coding region to generate a chimeric protein. Tissues from these mice may be explanted and placed in culture with luciferin to observe real time light emission as an indicator of PER2, and thus molecular clock oscillations (Yoo et al., 2004). To evaluate the effect of time of feeding on skeletal muscle rhythms, our lab limited access to food for only 4 hours/day for two weeks. The restricted feeding resulted in a shift in gene expression (PER2:LUC bioluminescence) in the skeletal muscle of the mice. In addition to studying the effect of time restricted feeding, our lab also demonstrated the ability of scheduled activity to entrain the skeletal muscle molecular clock (Wolff and Esser, 2012). Schedule bouts of either voluntary or involuntary endurance exercise resulted in a significant shift in clock gene expression (PER2:LUC bioluminescence) in 3 different muscle types as well as the lung. The shift in gene expression was observed in these tissues but not in the SCN, supporting a role for exercise as a non-SCN associated entrainment cue for skeletal muscle. Notably, the phase of the each of the three muscles, soleus, extensor digitorum longus (EDL) and flexor digitorum brevis (FDB), pre-exercise was distinct, highlighting the complexity of skeletal muscle and skeletal muscle circadian rhythms. This may be due to the differences in composition and function of the three muscles. The soleus is a postural muscle that is

comprised of mostly type I slow, oxidative fibers, whereas the EDL and FDB are intermittently recruited muscles (for either extension or flexion of the toes) and comprised of more type II fast oxidative-glycolytic and glycolytic fibers. Despite differences in the pre-exercise phase of the muscles, scheduled activity shifted the phases to about the same magnitude, 2-3 hours (Wolff and Esser, 2012). In addition, studies with the *Clock*$^{\Delta 19}$ mice have demonstrated decreases in protein levels of proliferator-activated receptor-γ coactivator-1 α (PGC1α) and mitochondrial transcription factor A with a concomitant decrease in mitochondrial content in skeletal muscle. Access to a running wheel under conditions of 12-hr:12-hr dark/light cycle and ad libitum access to food resulted in daily exercise (during the dark/active phase) in the clock mutant mice which partially rescued the metabolic phenotype of the skeletal muscle (Pastore and Hood, 2013). This illustrates the potential impact physical activity may have as an entrainment cue for skeletal muscle and also suggests that entrainment cues for skeletal muscle may be useful as therapies in conditions of circadian disruption.

The concept entrainment is important for this dissertation solely due to the fact that the mice used have impaired skeletal muscle circadian rhythms and therefore cannot be entrained to environmental cues. As a result, one can expect that skeletal muscle will not be able to anticipate and respond to the environment, such as metabolic changes (more specifically changes in glucose), and this may have detrimental effects on skeletal muscle metabolism, glucose homeostasis and metabolic health.

1.2.3 Synchrony of circadian rhythms in skeletal muscle and other metabolic tissues

Circadian research also demonstrates that synchrony of circadian rhythms in different tissues is important for normal physiology. This should be noted because synchrony of rhythms between metabolic tissues (skeletal muscle, liver, adipose) is necessary for normal metabolic function and health. A study by Yoo et. al. using the PER2:LUC mice demonstrated that peripheral (non-SCN) tissues had oscillators and that these oscillators had very distinct phases. Furthermore, they illustrated the importance of coordination of the tissue clocks by lesioning the SCN. In the presence of a lesioned SCN, the mice behave arrhythmically and the clocks in other tissues no longer express their normal phase relationships (Yoo et al., 2004).

As mentioned earlier, expression of genes involved in metabolism have been shown to oscillate in skeletal muscle. In fact, in the study by McCarthy et. al., one of the largest groups of oscillatory genes in skeletal muscle consisted of genes involved in substrate metabolism (McCarthy et al., 2007). Skeletal muscle and liver are considered key metabolic tissues and as such, it follows that coordination between these tissues would be critical for normal metabolic function at the systems level. A complete investigation of the coordination of the metabolic functions of these tissues in regards to circadian rhythms has not been done. However, data on the expression of circadian genes in skeletal muscle and liver has been collected and is available on the database CircaDB (Pizarro et al., 2013). Muscle and liver serve as tissues that participate in the regulation of blood glucose. From CircaDB one can see that genes involved in insulin signaling (*Irs1*, *Irs2*, *Akt2*, and *Tbc1d1*) and genes important for hepatic glucose production (*Pck1*, *G6pc*, *Pep* and *Pyg*) are circadian in both of these tissues and the phases are coordinated in a manner to facilitate blood glucose regulation (Table 1). Although protein and activity

Table 1. Circadian genes involved in carbohydrate homeostasis. Approximate time of peak expression in skeletal muscle and liver based on CircaDB (Pizarro et al., 2013). Dark gray represents the dark phase and light gray represents the light phase. Unhighlighted numbers represents borderline light/dark times.

	Skeletal Muscle	Liver Expression
	Peak	Peak
Akt2		~48
Irs1	~42	~24
Irs2	~30	~23
Tbc1d1	~42	~42
Insr		~30
Pck1		~37
Pep		~48
Pyg		~42
G6pc		~42

data is essential to confirm the gene data, it is apparent in Table 1 that during the light phase (or fasting phase for mice) genes involved in insulin signaling (*Irs1*, *Tbc1d1*, *Akt* and *Insr*) have reduced expression in both skeletal muscle and liver. During this time, genes involved in hepatic glucose production (*Pck1*, *G6pc*, *Pep* and *Pyg*) have increased expression. Furthermore, it has been shown in rodents that hepatic glucose production peaks during the fasting phase at which time insulin sensitivity is low (la Fleur et al., 2001; Matsumoto et al., 2007; Zhang et al., 2010). In contrast to the fasting phase, it is necessary for greater insulin sensitivity in the metabolic tissues during the feeding phase in order to take up and store post-prandial glucose (Oakes et al., 1997; Radziuk and Pye, 2001). Furthermore, the feeding phase is also often the active phase during which skeletal muscle requires more substrate. Therefore, during the feeding/active phase insulin sensitivity in liver and skeletal muscle needs to be higher (Figure 2). In this regard,

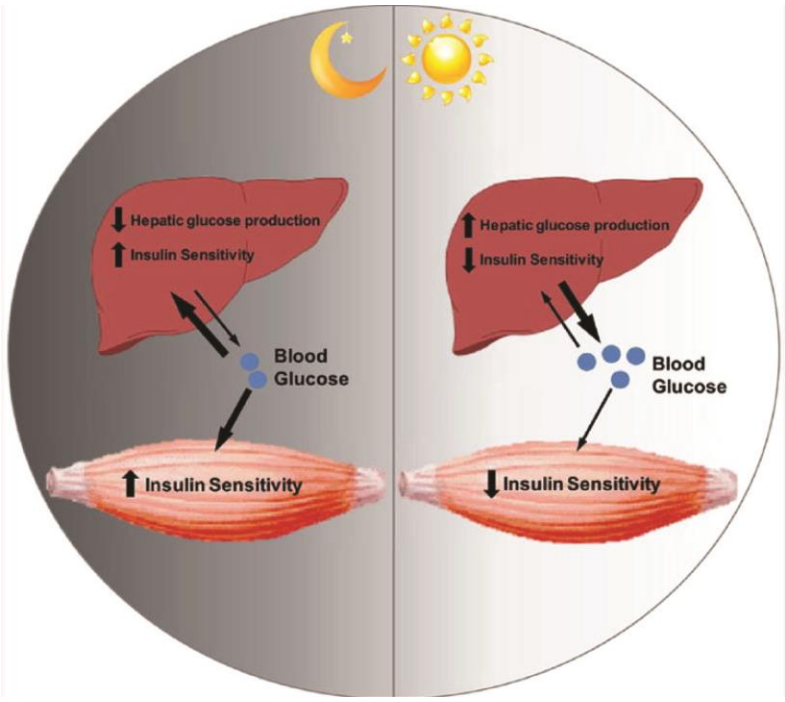


Figure 1.3. Cartoon depicting time-of-day coordination between skeletal muscle and liver metabolism for the regulation of systems glucose homeostasis. The dark phase/fed phase for mice is characterized by higher insulin sensitivity in skeletal muscle/liver and decreased hepatic glucose production. The light phase/fasting phase for mice is characterized by reduced insulin sensitivity in skeletal muscle/liver and increased hepatic glucose production.

synchrony between the skeletal muscle and other tissues is critical to normal physiological function.

The mice used in this dissertation have non-functional BMAL1 and have therefore lost skeletal muscle circadian rhythms. In the absence of skeletal muscle circadian rhythms, the skeletal muscle will lose the ability to synchronize rhythms with other metabolic tissues and this may contribute to impaired skeletal muscle metabolism and development of metabolic disorders. This will also be important later as some changes in the liver are seen despite the fact that *Bmal1* was only knocked down in skeletal muscle.

1.2.4 Skeletal muscle metabolism in circadian mutant models

The role of circadian clocks in regulating physiological processes has been largely studied through the use of genetic mouse models of clock disruption. These models include the *Per1/Per2* deficient mice, *Cry1/Cry2* double knockouts, *Clock*$^{-/-}$, *Clock*$^{\Delta 19}$ and *Bmal1*$^{-/-}$ mice. The first mouse model of clock disruption, the *Clock*$^{\Delta 19}$, was identified through a forward genetics screen and is a mouse model in which exon 19 (an exon critical for the DNA binding of the *Clock* gene) is mutated. These mice have a free running period of 28 hours, becoming behaviorally arrhythmic after 1-2 weeks in constant darkness (Vitaterna et al., 1994). *Clock*$^{\Delta 19}$ mice are moderately more susceptible to cancer and display a marked metabolic phenotype involving obesity, dyslipidemia, hepatic steatosis and hyperglycemia (Rudic et al., 2004; Turek et al., 2005; Lee et al., 2010). Interestingly, the *Clock*$^{-/-}$ mice (mice deficient of the *Clock* gene due to targeted gene knock down) do not exhibit the same phenotype as the *Clock*$^{\Delta 19}$ mice. *Clock*$^{-/-}$ display a slightly shorter period length with preserved behavioral rhythms. They have reduced lifespan, age-related cataract development and increased risk for dermatitis (Debruyne et al., 2006; DeBruyne et al., 2007; Dubrovsky et al., 2010). Differences between *Clock*$^{\Delta 19}$ and *Clock*$^{-/-}$ mouse models likely arise from the fact that the *Clock*$^{\Delta 19}$ mice have a dominant negative mutation in *Clock* whereas *Clock*$^{-/-}$ are deficient of *Clock* altogether and may have compensation from NPAS2. Models involving genes of the negative limb of the molecular clock, *Period* and *Cryptochrome,* appear to have less severe consequences in comparison to the *Clock*$^{-/-}$ and *Clock*$^{\Delta 19}$ mice. *Period* and C*ryptochrome* knockout mice do not display altered lifespan and the serious

physiological phenotype observed in *Clock*$^{\Delta 19}$ and *Bmal1*$^{-/-}$ mice. However, absence of Period does result in greater susceptibility to diet induced obesity (Yang et al., 2009).

Of the studied circadian mouse models, *Bmal1*$^{-/-}$ mice exhibit the most severe pathology. These mice display significantly reduced lifespan, behavioral arrhythmicity, ectopic calcification and sterility (Bunger et al., 2005; Kondratov et al., 2006; Yu and Weaver, 2011), and as such Bmal1 is the circadian gene that is more commonly targeted in to study circadian disruption.

Disruption of circadian rhythms is associated with an increased risk for development of metabolic disruption in both animals and humans (Karlsson et al., 2001; Rudic et al., 2004; Turek et al., 2005; Kroenke et al., 2007; Morikawa et al., 2007; Scott et al., 2008; Scheer et al., 2009; Marcheva et al., 2010). Besides the detrimental consequences of *Bmal* knockout on lifespan, bone health and arrhythmicity, *Bmal1*$^{-/-}$ mice show severe metabolic changes. These mice exhibit impaired insulin sensitivity, glucose tolerance and age-associated weight decline (Rudic et al., 2004; Kondratov et al., 2006; Andrews et al., 2010). As stated earlier, skeletal muscle comprises ~45% of the body mass of most mammals and is a critical component of normal metabolic health. Skeletal muscle is responsible for approximately 80% of postprandial insulin-mediated glucose disposal (DeFronzo et al., 1981; Ferrannini et al., 1988). In addition, altered muscle function can contribute to insulin resistance and metabolic syndrome (Kelley et al., 1999; Petersen et al., 2007). Our previous work in the germline *Bmal1*$^{-/-}$ mice demonstrated that *Bmal1*$^{-/-}$ and *Clock*$^{\Delta 19}$ mice display ~40 percent decrease in skeletal muscle mitochondrial volume. The mitochondria present in these mutant mice displayed aberrant morphology and increased respiratory uncoupling. Specifically a significant

reduction in state III respiration (ADP-stimulated, mmol O2/min/mg protein) in mitochondria isolated from gastrocnemius (GTN) muscle was observed (Andrews et al., 2010). There are limited studies on skeletal muscle specific *Bmal1* knockout mice and they demonstrate a significant impact on muscle metabolism as well. As mentioned previously, our lab showed that the most affected process in skeletal muscle of iMS*Bmal1*$^{-/-}$ mice is carbohydrate metabolism. In another study by Dyar et.al., muscle specific loss of *Bmal1* resulted in decreased skeletal muscle glucose uptake, reduced glucose oxidation, and increased PDK4 expression (Dyar et al., 2014). The increase in PDK4 expression coupled with an observed shift toward more oxidative fibers in the soleus muscle may indicate greater use of lipids for energy metabolism in the muscle of skeletal muscle specific *Bmal1* KO mice. These data demonstrate the importance the skeletal muscle molecular clock in the maintenance of systemic metabolic health. More importantly, they set the foundation of this dissertation by illuminating a role of the skeletal muscle clock in skeletal muscle glucose metabolism.

1.3 Skeletal muscle glucose metabolism

1.3.1 General background on glucose metabolism

Metabolism is a complex physiological function that includes all the chemical reactions of the body necessary to maintain life. This involves reactions in digestion, release of endocrine hormones, transport of substrates into cells, reactions used to produce cellular energy and much more (Marieb and Hoehn, 2012). Metabolism is essential for providing cellular energy, but also crucial in helping living organisms maintain homeostasis (Marieb and Hoehn, 2012; Wilson, 2013). For the purposes of this

dissertation it is important to understand glucose metabolism and glucose homeostasis. Glucose, a six-carbon hexamer, is one of the major energy substrates utilized by cells to produce ATP. Generally, when looking at dietary components, glucose is obtained from carbohydrates. These carbohydrates are catabolized into one of three simple sugars: glucose, fructose or galactose. Fructose and galactose can be converted to glucose in order to be metabolized via the glycolytic pathway. Glucose may also be obtained by gluconeogenesis or glycogenolysis. Gluconeogenesis is the conversion of fats and amino acids into glucose. Glycogenolysis is the breakdown of the highly branched storage form of glucose known as glycogen. Glucose is very strictly regulated in the body; blood glucose is maintained between 70-110 mg/dL of blood in humans. If glucose drops too low it can cause a range of symptoms from dizziness to seizures to even death. This can be attributed to the fact that certain tissue types, specifically neural tissue, relies almost entirely on glucose for metabolism. Therefore, when blood glucose is low, there is not enough energy substrate for neural tissue (such as the brain) to meet metabolic demands. In contrast, if blood glucose is elevated for too long it can lead to detrimental effects such as inflammatory responses, loss of fluid from cells (crenation as water osmosizes out of the cell to balance concentrations), build-up of atherosclerotic plaques over time and in severe cases stroke. As such, it is critical that the metabolism function normally to maintain glucose homeostasis. There are two well recognized metabolic states: the fed or postprandial state and the fasted state. In the postprandial state, after ingestion of a meal, blood glucose goes up and must be disposed of from the blood by metabolic tissues, generally as a result of insulin action. Insulin is released from the pancreas in response to elevated glucose and acts on tissues to increase glucose uptake, storage and utilization.

In the fasted state, blood glucose starts to drop and metabolism must work to bring it back up, usually through the release of anti-insulin acting hormones: glucagon, growth hormone, cortisol, epinephrine and norepinephrine (Beitner and Kalant, 1971; Marieb and Hoehn, 2012).

As previously stated, skeletal muscle is the largest metabolic organ in the non-obese individual and accounts for up to 80% of glucose metabolism in the postprandial state (DeFronzo et al., 1985; Ferrannini et al., 1985; Eckardt et al., 2014). Two of the metabolic processes in skeletal muscle that help maintain glucose homeostasis and provide cellular energy are glucose uptake and glycolysis. These two processes will be the main focus of this dissertation.

1.3.2 Skeletal muscle glucose uptake

Skeletal muscle glucose uptake is the process by which extracellular glucose is transported across the plasma membrane and into the skeletal muscle cell via facilitated diffusion. In skeletal muscle, glucose transport is mainly facilitated by glucose transporter type 4 (GLUT4) (Eguez et al., 2005). Uptake of glucose can be regulated in three distinct ways: delivery of glucose to skeletal muscle (circulation), transport of glucose across the membrane (presence and function of GLUT4), and utilization of glucose by skeletal muscle cells (glucose oxidation or glycolysis) (Wasserman et al., 2011; Richter and Hargreaves, 2013). This study concentrates on the latter two. In the basal state, GLUT4 is primarily located in intracellular stores and glucose transport across the membrane is minimal. Early studies demonstrate that GLUT4 localizes to a number of different intracellular compartments and organelles including the Golgi body,

trans-Golgi network, lysosomes, and late- and recycling-endosomes (Tanner and Lienhard, 1987; Kandror and Pilch, 1994; Hanpeter and James, 1995; Ralston and Ploug, 1996). There is some controversy over whether the intracellular pool of GLUT4 is static or if it is dynamic and constantly recycled between plasma membrane and the intracellular compartments, but regardless, membrane GLUT4 is kept nominal until stimulation. Upon stimulation, intracellular signaling occurs that results in translocation of GLUT4 to the cell membrane. The recognized triggers for GLUT4 translocation are insulin and contraction (Cushman and Wardzala, 1980; Constable et al., 1988; Douen et al., 1990; Goodyear et al., 1990; Brozinick et al., 1992; Czech and Buxton, 1993; Kanai et al., 1993; Cheatham and Kahn, 1995). Insulin and contraction signaling are two distinct pathways. This is known since contraction stimulated glucose uptake can occur normally in the presence of insulin resistance. However, they have some signaling molecules in common and both produce the same ultimate outcome, which is GLUT4 translocation to the membrane and increased glucose uptake (Ploug et al., 1987; Brozinick et al., 1992; Dolan et al., 1993; Lee et al., 1995; Kramer et al., 2006a; Kramer et al., 2006b; Taylor et al., 2008).

Insulin signaling initiates following binding of insulin to the insulin receptor (IR) (Cheatham and Kahn, 1995). The insulin receptor is a heterotetrameric complex consisting of two alpha subunits and two beta subunits. The alpha subunits bind insulin and the beta subunits possess tyrosine kinase activity. Insulin binding of the alpha subunits of the IR provokes trans-phosphorylation of specific tyrosines in the beta subunits which further increase the tyrosine kinase activity and allows particular molecules, insulin receptor substrates 1 and 2 (IRS1, IRS2) to dock. IRS1 and IRS2

contain Src homology (SH2) domains that recognize specific phosphotyrosine residues in the active IR. Once docked, IRS1 and IRS2 are phosphorylated and thus are able to interact with the next signaling molecule, phospho-inositol 3-kinase (PI3K) via its p85 regulatory subunit (Saltiel and Pessin, 2003; Watson et al., 2004). This interaction brings PI3K in proximity to the plasma membrane allowing it to convert phosphotidylinositol 4,5-bisphosphate (PIP_2) to phosphotidylinositol 3,4,5-triphosphate (PIP_3) (Shepherd, 2005). The presence of PIP_3 recruits molecules with pleckstrin homology (PH) domains – phosphoinositide dependent-kinase 1 (PDK1) and protein kinase B (AKT2) – to the membrane where PDK1 can phosphorylate and activate AKT2 (Corvera and Czech, 1998; Mora et al., 2004). AKT2 has many potential substrates that have been identified but only TBC1 domain family member 4 (TBC1D4 or AS160) and TBC1 domain family member 1 (TB1D1) have been studied in more detail. It is known that TBC1D1 and TBC1D4 inhibit GLUT4 translocation in the basal/unstimulated state. Translocation of GLUT4 to the membrane is controlled by Rab proteins, small proteins that are part of the Ras superfamily of monomeric G proteins. When bound to GTP, Rab proteins are active and facilitate trafficking of GLUT4 to the membrane. When bound to GDP they are inactive and GLUT4 translocation is low (Satoh, 2014). TBC1D1 and TBC1D4 have Rab-GTPase activity that regulates the activity of Rab proteins. Under basal conditions, TBC1D1 and TBC1D4 are active and cause the hydrolysis of GTP to GDP and inorganic phosphate rendering Rab proteins inactive. Insulin stimulation results in the phosphorylation of TBC1D1 and TBC1D4 by AKT2 which inactivates the Rab-GTPases. Therefore, Rab is bound to GTP and GLUT4 membrane trafficking occurs (Ramm et al., 2006; Satoh, 2014).

The signaling prompted by contraction begins in a manner distinct from insulin signaling. One of the major players in contraction-mediated glucose uptake is the protein AMP-activated protein kinase (AMPK). This protein is an energy sensor of the cell and becomes active in conditions of elevated AMP. When AMP is high, it binds AMPK causing a conformational change that exposes the catalytic domain. Active AMPK then phosphorylates TBC1D1/TBC1D4 to relieve inhibition of the translocation of GLUT4 by Rab proteins as described in insulin-signaling. In addition to AMPK, calcium ions and nitric oxide have also been suggested as contributors to increased glucose uptake with contractions. During skeletal muscle contraction, intracellular calcium increases and studies administering small doses of caffeine have shown that increased calcium leads to increased glucose uptake (Holloszy and Narahara, 1965). However, the mechanism by which calcium signals in skeletal muscle glucose uptake is still unknown. At this point in time, it is thought that calcium may work through activation of the calcium/calmodulin dependent protein kinases. Another possibility is that calcium effects are indirect and result from changes in AMP levels as the sarcoendoplasmic reticulum (SR) ATPase hydrolyzes ATP and pumps calcium back into the SR (Richter and Hargreaves, 2013). Regardless of the initial steps, outcome is inactivation of TBC1D1/TBC1D4 and translocation of GLUT4 from intracellular stores to the membrane (Figure 1.3).

It is evident that both insulin and contraction stimulation increase GLUT4 translocation and glucose uptake into the skeletal muscle. These signaling pathways are distinct as they begin signaling through different molecules and it is known that contraction-mediated glucose uptake can occur in the insulin-resistant condition (Brozinick et al., 1992; Cheatham and Kahn, 1995; Richter and Hargreaves, 2013). It is

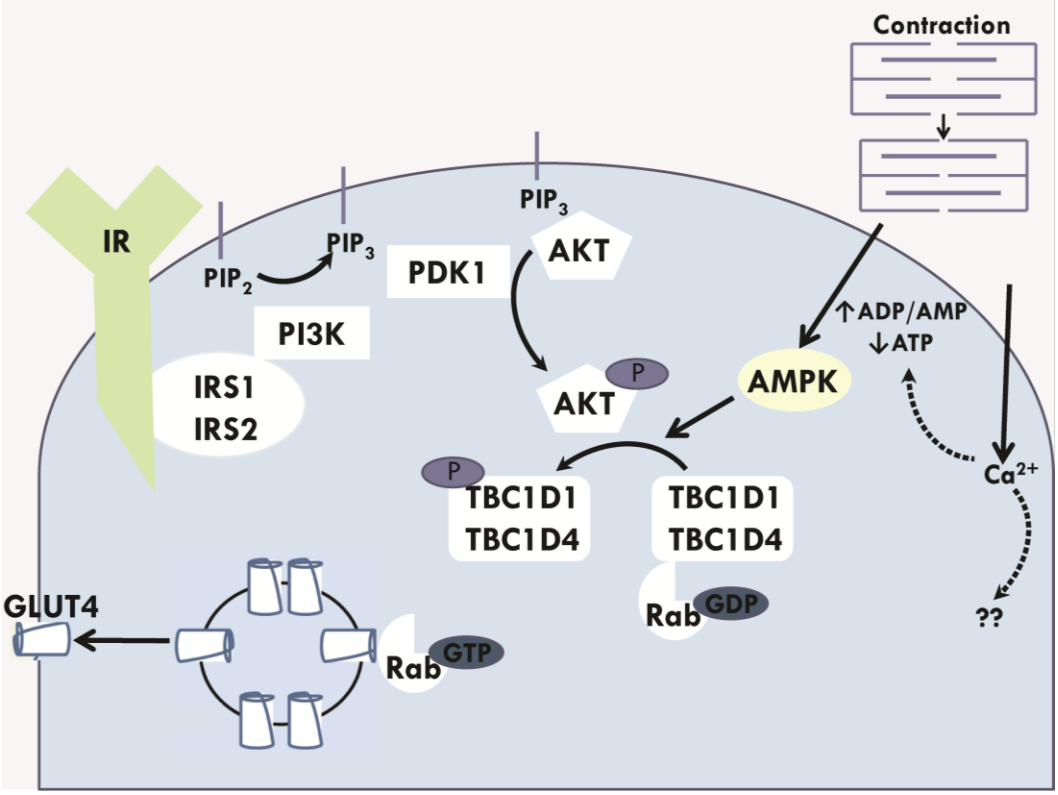


Figure 1.4. Diagram depicting insulin- and contraction-stimulated glucose uptake. Solid lines depict known mechanisms while dotted lines depict theorized mechanisms.

particularly important to note however, that despite differences in signaling, insulin- and contraction-stimulated glucose uptake both require GLUT4.

1.3.3 Skeletal muscle glycolysis

Glycolysis is an essential metabolic pathway in skeletal muscle for utilization of glucose as fuel. This pathway catabolizes glucose and converts it to pyruvate (in aerobic conditions) or lactic acid (in anaerobic conditions) while producing a net two ATP and two NADH. The glycolytic enzymes are present in the cytosol of the skeletal muscle cell, which is worthy to note since glycolysis helps establish the glucose concentration gradient between the extracellular space and the cytosol (Boiteux and Hess, 1981;

Marieb and Hoehn, 2012). This establishes a precarious link between glycolysis and glucose uptake. As glucose is taken up into the cell the first step of glycolysis occurs and glucose is immediately phosphorylated to glucose 6-phosphate by the key glycolytic enzyme hexokinase 2 (HK2) (Easterby and Qadri, 1982; Ritov and Kelley, 2001). The prompt phosphorylation of glucose maintains helps maintain glucose at a lower level inside the cell so that glucose can travel down its concentration gradient into the cell via GLUT4. If intracellular glucose gets too high, facilitated diffusion of glucose through the glucose transporter can no longer occur (Fueger et al., 2007). The reaction catalyzed by HK2 requires energy and therefore the hydrolysis of a molecule of ATP (Hanson and Fromm, 1965; Easterby and Qadri, 1982). Glucose 6-phophate is converted to fructose 6-phosphate, a pentamer sugar, by the enzyme glucose 6-phosphate isomerase/hexoglucose isomerase (HGI) (James and Notmann, 1973). Fructose 6-phosphate is recognized by a rate limiting glycolysis enzyme phosphofructokinase (PFK1) and using energy from hydrolysis of ATP PFK phosphorylates fructose 6-phosphate to fructose 1,6-bisphosphate (Bauer and Younathan, 1984; Seki et al., 2006). At this point the 6 carbons are split into two molecules containing 3 carbons each by aldolase. Aldolase breaks fructose 1,6-bisphosphate down into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (Hearn and Wainio, 1957; Mukai et al., 1984). The dihydroxyacetone phosphate must be converted into glyceraldehyde 3-phosphate in order to continue on in glycolysis. The conversion of dihydroxyacetone phosphate to glyceraldehyde 3-phosphate is accomplished by the enzyme triose phosphate isomerase (Boiteux and Hess, 1981). Both molecules of glyceraldehyde 3-phosphate are then converted to 1,3-bisphosphate by the removal of hydrogen catalyzed by 3-glyceraldehyde dehydrogenase. This reduction

oxidation reaction is coupled to the simultaneous reduction of NAD^+ to NADH (Vospelnikova et al., 1981; Bell et al., 2014). 1,3-bisphosphate is converted to 3-phosphoglycerate as 3-phosphoglycerate kinase removes the phosphate from the 2' carbon position and concomitantly synthesizes ATP from ADP. Two ATP are produced at this step for every molecule of glucose due to the breakdown of glucose into 3-carbon chains (Scopes, 1975; Zhou et al., 1991). The phosphate on the 3' position of 3-phosphoglycerate is moved to the 2' carbon position by phosphoglyceromutase generating 2-phosphoglycerate (Qiu et al., 2008). The enzyme enolase then catalyzes a dehydration reaction and phosphoenolpyruvate is obtained as water is removed (Haralambie and Reinartz, 1978; Merkulova et al., 2000). Pyruvate kinase dephosphorylates phosphoenolpyruvate to pyruvate while adding the phosphate to ADP to form ATP. As seen with the reaction a catalyzed by 3-phosphoglycerate kinase, two molecules of ATP are formed for each glucose catabolized (Baranowska and Baranowski, 1975) (Baranowska and Baranowski, 1977). Pyruvate is the main endpoint of glycolysis and can either be shuttled into the Kreb's cycle for oxidative metabolism or can be reduced to lactic acid by lactate dehydrogenase (Muirhead and Watson, 1992) (Figure 1.5).

The two main regulatory points for glycolysis in the skeletal muscle are the rate limiting enzymes HK2 and PFK1. HK2 is sensitive to the ratio of glucose 6-phosphate to glucose and is inhibited when glucose 6-phosphate is high. HK2 can greatly affect glycolytic rate and glucose uptake. If HK2 activity decreases, glucose will not be converted to glucose 6-phosphate for further glucose metabolism, and the concentration gradient promoting the movement of glucose into the cell will be lost

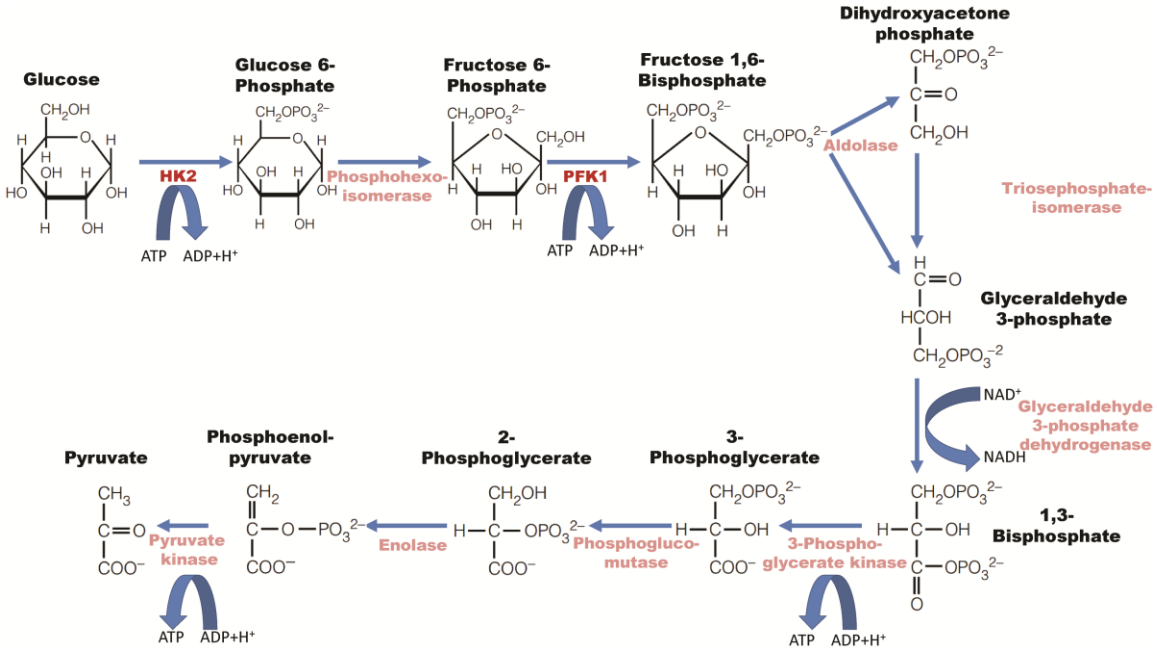


Figure 1.5. Basic schematic of the glycolytic pathway modified from Karp G, Cellular and Molecular Biology (2009), (Karp, 2009).

(Kruszynska et al., 1998; Fueger et al., 2007; Dieni and Storey, 2011). PFK1 activity is allosterically modulated and responds to the ADP:ATP ratio, changes in pH, creatine phosphate and citrate. PFK1 is often considered the major rate limiting enzyme of the glycolytic pathway (Tornheim and Lowenstein, 1976). PFK1 and HK2 activity are critical and decreased activity is often associated with insulin resistance and diabetes (Bauer and Younathan, 1984; Vestergaard et al., 1993; Sanderson et al., 1996; Kruszynska et al., 1998; Vestergaard, 1999; Fueger et al., 2007). Due to their significant impact on glycolytic flux and the role of HK2 in glucose uptake, these two enzymes will be a focus of this dissertation. The metabolites generated by glycolysis will also be important.

1.4 The whole picture: circadian rhythms, glucose uptake and glycolysis.

Circadian rhythms generate the approximate 24 hour oscillations in living organisms that control physiological functions such as metabolism. These rhythms are generated by a molecular clock that in mammals consists of core molecular clock genes, *Bmal1, Clock, Per1/2* and *Cry1/2* (Shearman et al., 2000; Buhr and Takahashi, 2013; Robinson and Reddy, 2014). Virtually all cells in the body have a molecular clock that both acts as a time-keeping mechanism and directs some tissue-specific gene expression (Panda et al., 2002; Yoo et al., 2004; Kornmann et al., 2007; Shostak et al., 2013; Hodge et al., 2015b). When the clock mechanism is disrupted it leads to detrimental effects, one major effect being development of metabolic disease. Studies in clock disrupted models demonstrate impairments in glucose tolerance, insulin sensitivity, and body weight and composition (Rudic et al., 2004; Turek et al., 2005; Kondratov et al., 2006; Scheer et al., 2009; Lee et al., 2013). However, the contribution of tissue-specific clocks to metabolism is yet unclear. Our lab recently identified 1,628 circadian mRNAs in skeletal muscle; gene ontology analysis that 62% of those 1,628 mRNAs had metabolic functions. Furthermore, when looking at changes in gene expression of muscle from skeletal muscle specific *Bmal1* knockout mice we discovered that carbohydrate metabolism was the most affected process. This suggests a vital role for the skeletal muscle clock in glucose metabolism (Hodge et al., 2015b).

Glucose metabolism is essential for providing energy for cells and maintaining homeostasis. Dysfunctions in glucose metabolism can easily lead to metabolic problems including blood glucose issues, changes in insulin sensitivity, glucose intolerance and diabetes (Daly, 2003; Marieb and Hoehn, 2012). Skeletal muscle is a crucial metabolic tissue and accounts for up to 80% of glucose metabolism in the postprandial stated.

Accordingly, skeletal muscle glucose metabolism has a significant impact on metabolic health (DeFronzo et al., 1985; Ferrannini et al., 1985). Glucose metabolism involves a variety of processes: two major processes in skeletal muscle being glucose uptake and glycolysis. Glucose uptake into skeletal muscle is stimulated by insulin and contraction, which rely on distinct signaling pathways, but both depend on the skeletal muscle glucose transporter GLUT4 (Kanai et al., 1993; Deems et al., 1994; Ren et al., 1994; Richter and Hargreaves, 2013). Skeletal muscle uptake of glucose helps clear blood glucose to maintain set point (70-110 mg/dL) and provides skeletal muscle with glucose as an energy substrate. After uptake, glucose can be metabolized by skeletal muscle through the glycolysis, an 11-12 step pathway involving key enzymes such as HK2 and PFK1 (Boiteux and Hess, 1981).

Our understanding of the skeletal muscle clock's role in metabolism is limited, but as stated, loss of the skeletal muscle clock significantly alters expression of mRNAs involved in carbohydrate metabolism (Hodge et al., 2015b). In addition, a recent study by Dyar and colleagues provides impelling evidence that the skeletal muscle clock helps regulate glucose metabolism, as loss of skeletal *Bmal1* resulted in impaired glucose uptake, decreased GLUT4 levels, reduced HK2 levels and diminished PDH activity (suggesting a possible shift from glucose to fatty acid metabolism) (Dyar et al., 2014). Much of these results were done solely in a non-inducible model though, so may be complicated by developmental effects. This dissertation further investigates the impact of skeletal muscle specific clock dysfunction using our own inducible skeletal muscle specific *Bmal1* knockout mice (iMS*Bmal1*$^{-/-}$). The aims of the study were to determine how loss of skeletal muscle Bmal1 affected glucose uptake, glycolytic flux and the

overall body metabolic phenotype. This was accomplished using a combination of ex vivo radiolabeled glucose uptake experiments, RT PCR, western blot, enzyme activity assays, blood analysis, echo-MRI and tolerance tests. Results will be presented demonstrating impairment in glucose uptake, decreased glycolytic flux and altered phenotype (e.g. changes in body composition, altered blood glucose/insulin and impaired glucose tolerance). The data obtained expands our knowledge of skeletal muscle clock function in glucose metabolism, provides data on inducible muscle specific mice not included in the Dyar paper and notably demonstrates a more profound metabolic phenotype compared to the skeletal muscle specific Bmal1 knockout mice in the Dyar study (Dyar et al., 2014).

CHAPTER 2: Methods

Ethical Approval

All experimental procedures were done in accordance with the institutional guidelines for the care and use of laboratory animals and approved by the University of Kentucky Institutional Animal Care and Use Committee.

Animal Care and Use

Inducible skeletal muscle specific *Bmal1* knockout mice were generated as previously described (Hodge et al., 2015b). We obtained the skeletal muscle specific, tamoxifen inducible Cre recombinase mouse from the Center for Muscle Biology at the University of Kentucky. This mouse has a Cre recombinase flanked by two mutated estrogen receptors and a human skeletal actin promoter. Past work has been published confirming the efficacy of this mouse for muscle specific gene recombination in adult mice (McCarthy et al., 2012). The tamoxifen inducible Cre recombinase mouse was crossed with the *Bmal1* floxed mouse acquired through Jackson Labs (B6, 1294(Cg)-*Arntl*$^{tm1Weit/J}$), to generate the inducible skeletal muscle specific *Bmal1* floxed mouse (iMS*Bmal1*$^{fl/fl}$). After 12 weeks of age, the iMS*Bmal1*$^{fl/fl}$ mice injected intraperitoneally with 2g/kg of either vehicle or tamoxifen for 5 consecutive days. The age of injection was chosen in attempt to eliminate any developmental effects. Recombination has been confirmed and is demonstrated in Hodge et. al (Hodge et al., 2015b). Prior to experiments mice were housed in 14:10, light:dark conditions. All experiments were performed 5 weeks post-recombination. All experiments, with the exception of the non-fasting blood

glucose, behavior measurements and echo-MRI, were done 2 hours after the lights turned off. This time was chosen due to the fact that glucose tolerance is higher in the beginning of the active phase (la Fleur et al., 2001). Both genders were used with a higher ratio of male to female mice. Echo-MRI was also performed at 12 weeks post-recombination. Mice were euthanized prior to glucose uptake experiments and tissue collections. Euthanization was done by anesthetizing mice with isoflurane followed by cervical dislocation.

Echo-MRI

Body composition was quantified in conscious mice at both 5 and 12 weeks post-recombination using EchoMRI Quantitative Magnetic Resonance Body Composition Analyzer (Echo Medical Systems, Houston, Texas) (n=7/group). Before body composition analysis, mice were weighed. Conscious mice were then placed into a measuring tube of appropriate size and the tube was placed into the Echo-MRI machine which provided data on fat mass, lean mass and water mass through detection of distinctions in NMR amplitudes of the different tissues.

Glucose tolerance test

Glucose tolerance tests (n=8) were performed two hours after lights turned off, a time at which glucose tolerance is highest (la Fleur et al., 2001). Mice were fast six hours prior to the start of the test and all measurements were done with an AlphaTrak glucometer (Abbott Animal Health). A measurement was taken prior to injection of a bolus of

glucose (0 minutes). Mice were injected intraperitoneally with 2mg/kg glucose and additional measurements were taken at 15, 30, 60, 90, and 120 minutes post-injection.

Insulin sensitivity assays

Mice were fasted for six hours prior to tests, such that the start of the experiment coincided with zeitgeber time 14 (two hours past lights out) (n=8). Three different doses of insulin were tested: a submaximal dose of 0.75 U/kg, a maximal dose of 1.0 U/kg and an intermediate dose of 0.85 U/kg. At the start of the experiment (time point 0), the tail vein was nicked and blood glucose was measured using an AlphaTrak glucometer. Mice were then injected with the appropriate dose of Humulin R-100 insulin. Blood glucose was monitored by further measurements at 15, 30, 45 and 60 minutes post injection. If blood glucose dropped below 70 mg/dL or mice began showing signs of low blood glucose, they were immediately injected with a bolus of dextrose.

Blood measurements

All fasting blood measures were obtained after a 6 hour fast. Fasting blood glucose (n=8) was measured with the AlphaTrak glucometer and fasting insulin (n=7/group) was measured by collecting blood from the tail vein of the mice and running a colorimetric ELISA (Crystal Chem). Non-fasting blood glucose was measured by measuring blood glucose every 4 hours for 24 hours and averaged (n=4 per time point).

Glucose uptake experiments

All solutions in this experiment were bubbled with 95%O_2/5%CO_2 and kept at 35°C. Extensor digitorum longus muscles were excised from each leg of the mice and placed in a recovery media (Krebs Henseleit Buffer (KHB) with 0.1% bovine serum albumin, 2 mM sodium pyruvate, 6 mM mannitol) with or without 2,000 µU/mL insulin. After 30 minutes, muscles were transferred to an incubation buffer (KHB with 0.1% bovine serum albumin, 2 mM sodium pyruvate, 6mM mannitol, 1 mM 2-deoxy-D-glucose, 2.25 µL/mL [3H]-2-deoxyglucose, 2 µL/mL [14C]-mannitol) with or without 2000 µU/mL insulin (consistent with what the muscle was exposed to in recovery media) for exactly 20 minutes. After 20 minutes, tissues were flash frozen. Tissue lysates were prepared and 100 µL of each sample was added to a scintillation vial with 5 mL of scintillation fluid followed by radioactivity measurement in a scintillation counter.

Real time PCR (RT-PCR)

RNA was isolated from gastrocnemius samples of iMS*Bmal1*$^{+/+}$ and iMS*Bmal1*$^{-/-}$ mice (n=7/group). Briefly, 50-100 mg of gastrocnemius tissue was homogenized in 1 mL Trizol (Invitrogen). Phase separation, RNA precipitiation and RNA washes and RNA redissolving were carried out as per manufacturer's instructions. RNA was quantified through spectrophotometry with wavelength, $\lambda = 260$ nm. Total RNA was then used to synthesize cDNA using a mixture of oligo(dT) primer and random hexamers in SuperScript III First-Strand Synthesis SuperMix (Invitrogen, Waltham, MA, USA) kit. Expression of *Glut4*, *Hk2*, and *Pfk1* was done using cDNA and the following Taqman primers: Mm01245502_m1, Mm00443385_m1, Mm01309576_m1, Mm99999915_g1

and Mm02343715_g1. Gene expression was determine using the $2^{\wedge-\Delta\Delta C_T}$ method and all target genes were normalized using *Gapdh* or *Rpl26*.

Circadian Microarray

Inducible skeletal muscle specific mice (iMS*Bmal1*$^{+/+}$ and iMS*Bmal1*$^{-/-}$) were housed in light boxes and entrained to a 12:12 light dark cycle for five weeks. During this experiment mice were given ad libitum access to both food and water. 30 hours prior to the start of collections, mice were released into constant darkness. Samples (including gastrocnemius muscle, liver and blood) were collected every 4 hours for 28 hours. RNA was isolated from gastrocnemius samples as previously described. Equal amounts of RNA was pooled from the four mice in each group at each time point. cDNA libraries were generated from the pooled RNA samples and these libraries were then hybridized to Affymetrix mouse gene 1.0 ST microarrays (Affymetrix, Santa Clara, CA). Relative intensity values were obtained and analyzed as described in Hodge et.al., 2015 (Hodge et al., 2015b).

Western blot analysis

Tissue lysates were made from gastrocnemius muscles of iMS*Bmal1*$^{+/+}$ and iMS*Bmal1*$^{-/-}$ mice. Proteins were separated by SDS-PAGE using 4-15% Tris-HCl precast gels (BioRad), transferred and immunoblotted using routine methods. GLUT4 was detected using a primary monoclonal GLUT4 antibody (Cell Signaling, #2213) and an AlexaFluor680 goat anti-rabbit secondary antibody (Invitrogen, #A-21109).

Hexokinase activity assay

Hexokinase activity was measured following as previously described.(Scheer et al., 1978) Gastrocnemius tissue (n=7/group) was homogenized in a buffer (1:10, weight:volume) containing 150 mM KCl, 10 mM $MgCl_2$, 5 mM EDTA and 5 mM β-mercaptoethanol. Samples were centrifuged at 15,000xg for 1 hour while experimental solutions, A (47 mM Tris (pH 7.4), 10 mM $MgCl_2$, 0.8 mM NADP, 0.5 mM glucose, 5.0 mM mercaptoethanol, 0.1 units glucose 6-phosphate dehydrogenase), and B, were prepared(47 mM Tris (pH 7.4), 10 mM $MgCl_2$, 0.8 mM NADP, 0.5 mM glucose, 5.0 mM mercaptoethanol, 0.1 units glucose 6-phosphate dehydrogenase, 5 mM ATP, 0.27 mM phosphoglyceric acid). An eppendorf tube containing 2.45 mL of A or B was made for each tissue sample. After centrifugation was complete, 0.05 mL of tissue sample was added to each tube. Absorbance was measured at 30°C and 340 nM every 2 seconds for 10 minutes. Samples were measured in duplicate. As the glycolytic reaction takes place, NADP is oxidized to form NADPH which has an absorbance at 340 nm. Activity is shown as μM/g/min (micromole NADPH formed per gram of tissue per minute)

Phosphofructokinase activity assay

The same tissue samples used for the hexokinase activity assay were used for the phosphofructokinase assay but a different reaction mixture/solution was used. The reaction mixture for this assay contained 50 mM Tris-HCl (pH 8), 1 mM EDTA, 6 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.16 mM NADH, 1 mM ATP, 1 mM fructose-6-phosphate, 0.4 units aldolase, 2.4 units triose-phosphate isomerase and 0.4 units α–glycero-phosphate dehydrogenase. 5 μL of tissue sample was added to 295 μL of reaction

mixture and absorbance was read at 25°C and 340 nM for 10 minutes. As the glycolytic reaction takes place, NADH, which is detectable at 340 nm, is reduced to NAD^+ and absorbance decreases. Activity is shown in U/g/min (where U is enzyme activity based upon the reduction of NADH).

Glycogen Assays

Glycogen concentrations were determined for gastrocnemius tissue and liver using samples from the circadian time course collection (n= 32). Approximately 30-50 mg of tissue was cut and weighed. Samples were immersed in 1 mL of 30% KOH saturated with Na_2SO_4 and boiled at 95°C for 20-30' until complete digestion. Boiled samples were vortexed and placed on ice until cool. Glycogen was then precipitated by adding 2 mL of 95% ethanol. Precipitated samples were vortexed and incubated another 30 minutes on ice. After the incubation, samples were centrifuged at 550Xg for 30 minutes. The supernatant was decanted and the tubes were dried upside down for five minutes or until dry. The pellet was redissolved in 1 mL of water and vortexed until completely in solution. A milliliter of 5% phenol was added followed by 5 mL of 96% H_2SO_4. The mixture was incubated on ice for 30 minutes as color developed and then absorbance was read at 490 nm. Concentrations were determined using a concentration curve with glycogen standards (0, 25, 50, 75 and 100 g/ml glycogen).

Metabolomics data

Gastrocnemius muscles were obtained from anesthetized mice using liquid nitrogen cooled clamps. Tissue was immediately flash frozen and labeled gastrocnemius samples

were sent to University of Michigan Metabolomics Core Services for processing. For metabolomics, 20mg of gastrocnemius tissue was ground up and transferred to a microtube containing isotope-labeled internal standards and a mixture of methanol, chloroform and water (8:1:1). Samples were sonicated (40% power output, 20% duty cycle, 20 seconds), chilled (4°C, 10 minutes) and centrifuged (4°C, 14,000 RMP, 10 minutes). Liquid chromatography-mass spectrometry analysis was performed on an Agilent system with a 1260 UPLC module coupled to a 6520 Quadrupole Time-of-Flight spectrometer (Agilent Technologies, CA). Data obtained was processed with MassHunter Quantitative Analysis version B.07.00. Sample data was normalized to the nearest isotope-labeled standard.

Statistics

For statistics on blood concentrations, glucose tolerance, RT-PCR, body composition, behavior and enzyme activities and student t-test was utilized. 2-way ANOVA was used to evaluate data from glucose uptake experiments (comparing time exposed to insulin and genotype). Metabolites from the glycolytic pathway were analyzed using a Hotelling's T-squared test to identify if metabolites within the same pathway were changed between iMS*Bmal1*$^{+/+}$ and iMS*Bmal1*$^{-/-}$ mice.

Chapter 3: Results

Previous studies in *Bmal1* knock out mouse models have demonstrated that loss of *Bmal1* significantly affects systemic metabolic parameters (Rudic et al., 2004; Kennaway et al., 2013). In order to assess the contribution of the molecular clock in skeletal muscle to systemic metabolic homeostasis, we generated an inducible skeletal muscle specific *Bmal1* knock out mouse (iMS*Bmal1*$^{-/-}$; following tamoxifen treatment) for the targeted disruption of the molecular clock mechanism only in adult skeletal muscle.(Hodge et al., 2015a) As early as 5 weeks post-treatment, iMS*Bmal1*$^{-/-}$ mice demonstrated an increase in lean composition and a trend towards reduced fat composition relative to iMS*Bmal1*$^{+/+}$ (**Figure 3.1A**). These changes became more pronounced at 12 weeks post treatment, at which point, iMS*Bmal1*$^{-/-}$ mice were leaner, had less fat composition and displayed an overall decrease in body weight compared to iMS*Bmal1*$^{+/+}$ mice (**Figure 3.1B**). These changes in body composition could not be attributed to altered feeding or behavior, as there were no detectable differences in net feeding or net activity in the iMS*Bmal1*$^{-/-}$ mice (**Figure 3.1C**). The observed changes in body composition, coupled with the lack of difference in net feeding and activity, suggested differences in metabolic fuel usage. For this reason, we performed glucose tolerance tests, as well as, assessed blood glucose and insulin. Fasting blood glucose (**Figure 3.2A**) was not significantly different between groups, but iMS*Bmal1*$^{-/-}$ mice had elevated fasting insulin levels compared to iMS*Bmal1*$^{+/+}$ mice (**Figure 3.2B**). When injected with a bolus of exogenous glucose, iMS*Bmal1*$^{-/-}$ mice exhibited a greater overall increase in blood glucose. In addition, glucose levels

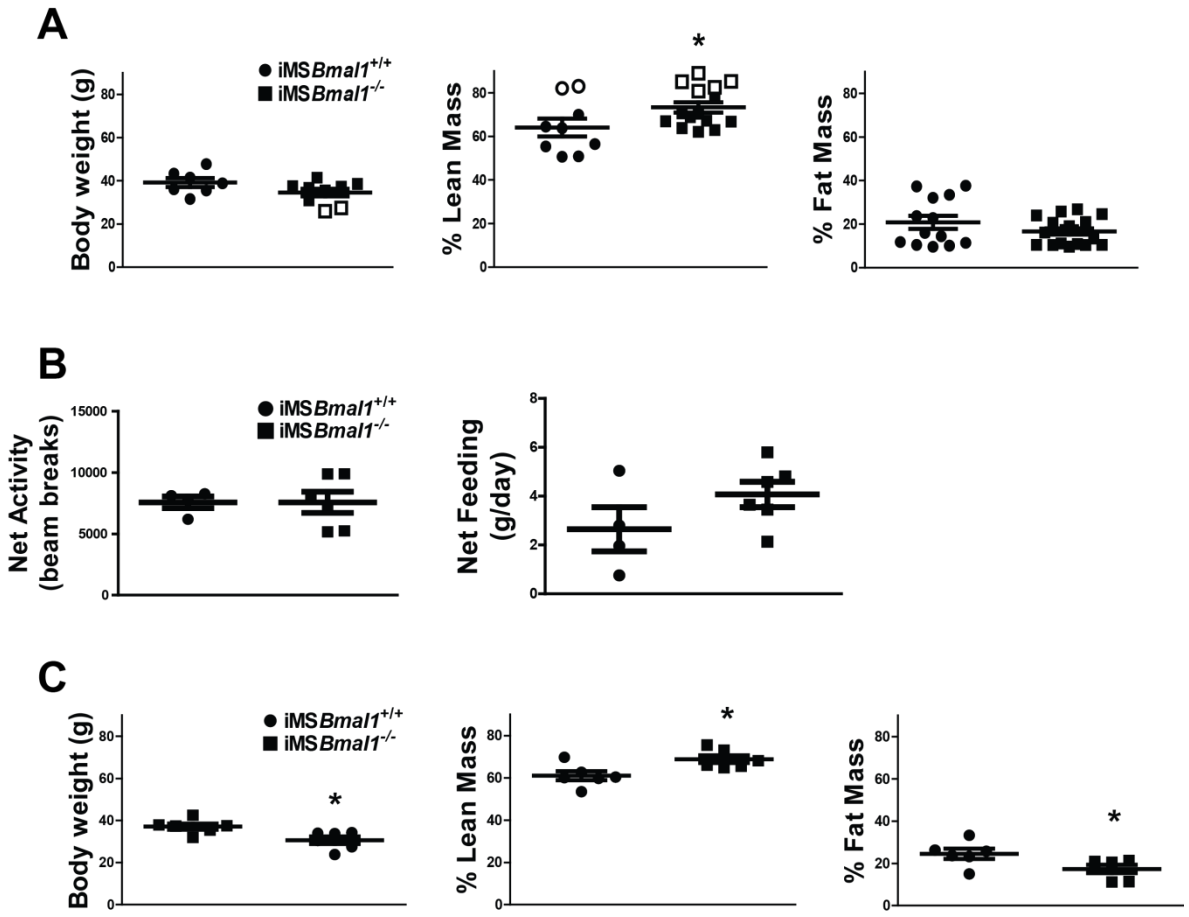


Figure 3.1. Body composition (including body weight, % lean mass, % fat mass and %water weight) and behavior (including net feeding and net activity) are shown for the iMS*Bmal1*$^{+/+}$ and iMS*Bmal1*$^{-/-}$ mice. (A) Body weight and composition in mixed genders at 5 weeks post-recombination (n=13 iMS*Bmal1*$^{+/+}$, n=18 iMS*Bmal1*$^{-/-}$). (B) Body weight and composition for exclusively male mice at 5 weeks post-recombination (n=13 iMS*Bmal1*$^{+/+}$, n=16 iMS*Bmal1*$^{-/-}$). (C) Body weight and composition at 12 weeks post-recombination (n=6 iMS*Bmal1*$^{+/+}$, n=7 iMS*Bmal1*$^{-/-}$). (D) Feeding and activity over 4 days at approximately 5 weeks post-recombination (n=4 iMS*Bmal1*$^{+/+}$, n=6 iMS*Bmal1*$^{-/-}$).

remained elevated for a longer period of time in the iMS*Bmal1*$^{-/-}$ mice. The combination of the overall increase in blood glucose, and the extended time that blood glucose remained elevated in the iMS*Bmal1*$^{-/-}$ mice, significantly increased area under the curve when compared to the control mice (iMS*Bmal1*$^{+/+}$) (**Figure 3.2C**). We carried out a circadian time course collection with vehicle and tamoxifen-treated mice. Blood from iMS*Bmal1*$^{+/+}$ and iMS*Bmal1*$^{-/-}$ mice were collected every 4 h over a 28-h period, and

non-fasting blood glucose was measured. When blood glucose was measured in the non-fasting state, iMS*Bmal1*$^{-/-}$ displayed significantly higher blood glucose values over a 24 hour period of time relative to iMS*Bmal1*$^{+/+}$ mice (**Figure 3.2D)**.

Skeletal muscle is the major site for glucose disposal in the postprandial state. The measured glucose intolerance and elevated non-fasting blood glucose levels implied a defect in skeletal muscle glucose uptake. We examined this further by measuring both insulin-stimulated and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR)-stimulated glucose uptake in the extensor digitorum (EDL) muscles of iMS*Bmal1*$^{+/+}$ and iMS*Bmal1*$^{-/-}$ mice. Both insulin and muscle contraction have been demonstrated to stimulate glucose uptake into the skeletal muscle. AICAR allows for the examination of contraction stimulated glucose uptake. We found that insulin-stimulated glucose uptake was significantly impaired in the iMS*Bmal1*$^{-/-}$ mice (**Figure 3.3A**). When EDL muscles were stimulated with AICAR in place of insulin, iMS*Bmal1*$^{-/-}$ still exhibited a significant decrease in glucose uptake (**Figure 3.3B**).

Insulin and AICAR stimulate separate pathways. However, our results indicated that a component essential to both pathways contributed to the observed impairment of glucose uptake. Both pathways rely on the translocation of the glucose transporter (*Glut4*) to the plasma membrane to allow transport of glucose from blood into the cell. In the iMS*Bmal1*$^{-/-}$, both mRNA expression and protein content of the glucose transporter were dramatically reduced relative to that measured in iMS*Bmal1*$^{+/+}$ mice (**Figure 3.4 A,B**).

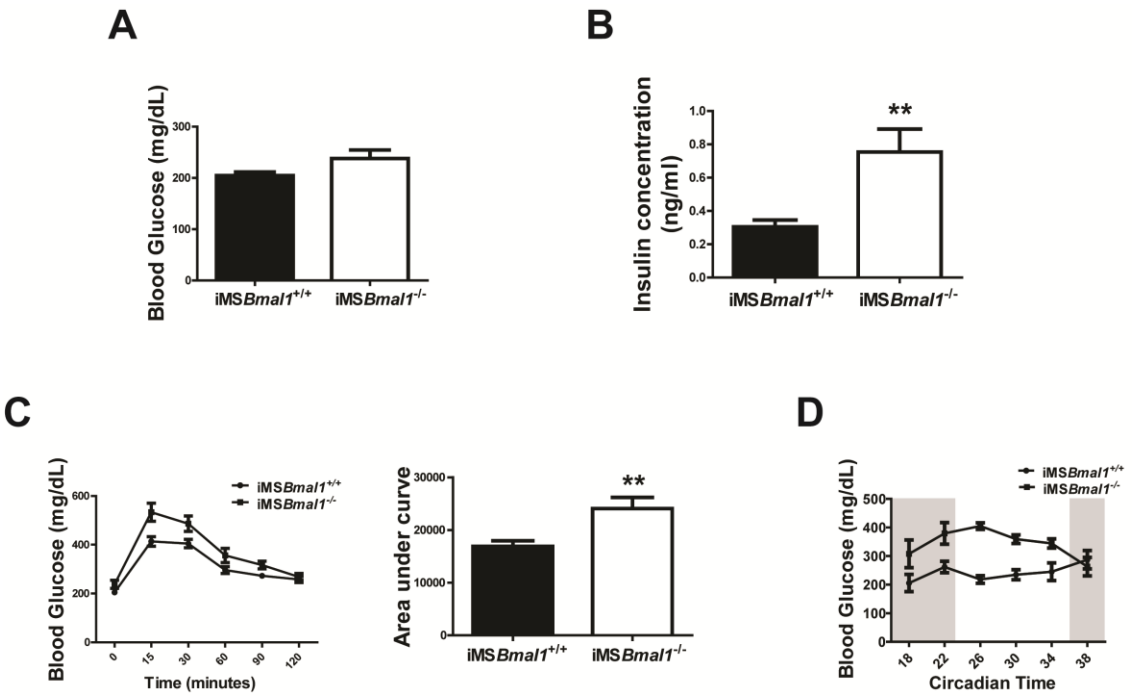


Figure 3.2. Fasted blood glucose and insulin, glucose tolerance and non-fasted blood glucose for the iMS*Bmal1*$^{+/+}$ and iMS*Bmal1*$^{-/-}$ mice at 5 weeks post-recombination. (A) Fasted blood glucose (n=8). (B) Fasted blood insulin (n=7/group). (C) Glucose tolerance depicted as blood glucose versus time post-glucose injection and area under the curve (n=8). (D) Non-fasted blood glucose measured every 4 hours for 24 hours (n=24). Gray blocks indicate the active phase and white areas indicate the inactive phase.

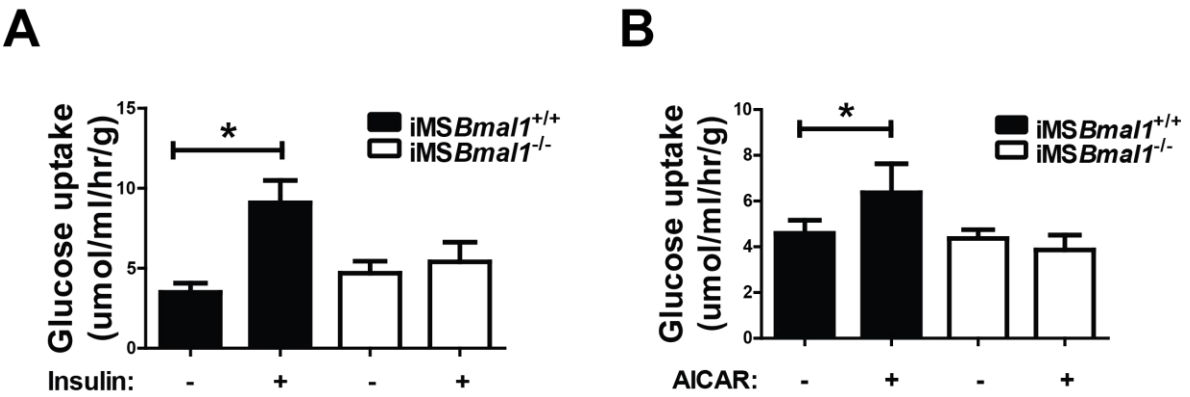


Figure 3.3. Glucose uptake (both insulin- and AICAR-stimulated) in the iMS*Bmal1*$^{+/+}$ and iMS*Bmal1*$^{-/-}$ mice. (A) Insulin stimulated glucose uptake in the EDL muscles using a maximum insulin dose and 20 minutes of exposure (n=8). (B) AICAR stimulated glucose uptake in the EDL muscles after 40 minutes of exposure incubation media with or without AICAR (n=5).

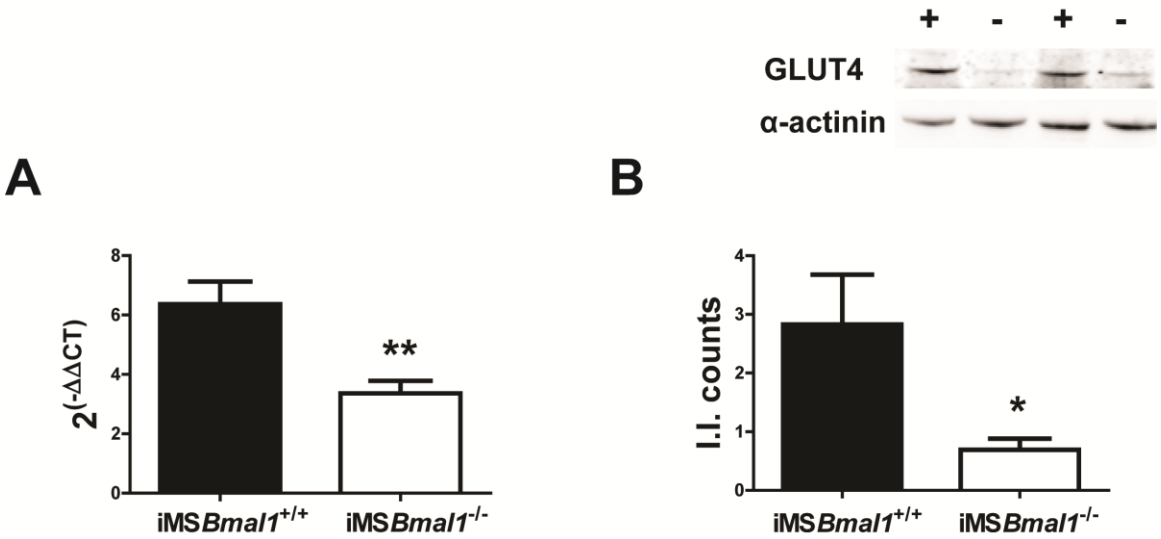


Figure 3.4. mRNA expression and protein content of the glucose transporter type 4 (*Glut4*/GLUT4) in the iMS*Bmal1*$^{+/+}$ and iMS*Bmal1*$^{-/-}$ mice. (A) mRNA expression of *Glut4* in the gastrocnemius muscle measured using RT PCR and calculated via the $2^{(-\Delta\Delta CT)}$ method (n=6). (B) Protein content of GLUT4 in the gastrocnemius muscle was measured as integrated intensity (I.I. counts) of the fluorescent bands (n=7/group).

Upon entering the cell, glucose is phosphorylated to glucose-6-phosphate by the glycolytic enzyme hexokinase 2 (HK2). This transformation of glucose allows the cell to maintain a glucose gradient relative to the extracellular fluid to sustain glucose influx. In this regard, HK2 activity may affect glucose uptake. Microarray analysis was done on gastrocnemius tissue from the aforementioned circadian time course collection. Signal intensity measured for *Hk2* mRNA was significantly lower in the iMS*Bmal1*$^{-/-}$ mice (**Figure 3.5A**). This suggests lower expression of *Hk2* in the iMS*Bmal1*$^{-/-}$ mice which is consistent with the decreased protein content demonstrated in the Dyar study (Dyar et al., 2014). This does not however, indicated activity levels of the HK2 enzyme so we went a step further and measured HK2 activity in the gastrocnemius muscles of the iMS*Bmal1*$^{+/+}$ and iMS*Bmal1*$^{-/-}$ mice. HK2 was found to have significantly reduced enzymatic activity in iMS*Bmal1*$^{-/-}$ mice providing strong evidence that glucose phosphorylation is diminished (**Figure 3.5B**). Besides having an impact on glucose

uptake, HK2 is a rate limiting enzyme in the glycolytic pathway. In order to further evaluate glycolytic flux in the iMSBmal1$^{-/-}$ mice, we examined another rate limiting enzyme of glycolysis, phosphofructokinase (PFK1). *Pfk1* mRNA expression was reduced in iMS*Bmal1*$^{-/-}$ mice and PFK1 activity was significantly reduced as well (**Figure 3.5 C, D**). To further examine glycolytic flux we assessed metabolite profiles in the gastrocnemius of the iMS*Bmal1*$^{+/+}$ and iMS*Bmal1*$^{-/-}$ mice. Metabolomics data revealed elevated glucose within the muscle of the iMS*Bmal1*$^{-/-}$ mice compared to the iMS*Bmal1*$^{+/+}$ mice (**Figure 3.6A**). Excess glucose is stored in muscle as the multi-branched, polysaccharide glycogen. Consistent with the observed increased muscle glucose from the metabolomics data we observed an increase in glycogen in the gastrocnemius muscle of the iMS*Bmal1*$^{-/-}$ mice (**Figure 3.6B**). This suggests that glucose is being stored in skeletal muscle rather than being oxidized. This data is congruous with the observed decrease in activity of rate-limiting glycolytic enzymes, PFK1 and HK2, but to strengthen our investigation we also looked at glycolytic metabolites. Two glycolytic metabolites, glyceraldehyde 3-phosphate and phosphoenolpyruvate trended to be lower while both 2- and 3-phopshoglycerate displayed a significant reduction in the iMS*Bmal1*$^{-/-}$ mice. We evaluated glycolytic metabolites from the microarray dataset as a group within each genotype using the Hotelling's t-squared test for independent samples. Results indicated that as a group, these metabolites were significantly decreased in the iMS*Bmal1*$^{-/-}$ mice relative to iMS*Bmal1*$^{+/+}$ mice (**Figure 3.6C**). This evidence reinforces the idea that oxidation of glucose is diminished in the skeletal muscle of iMS*Bmal1*$^{-/-}$ mice.

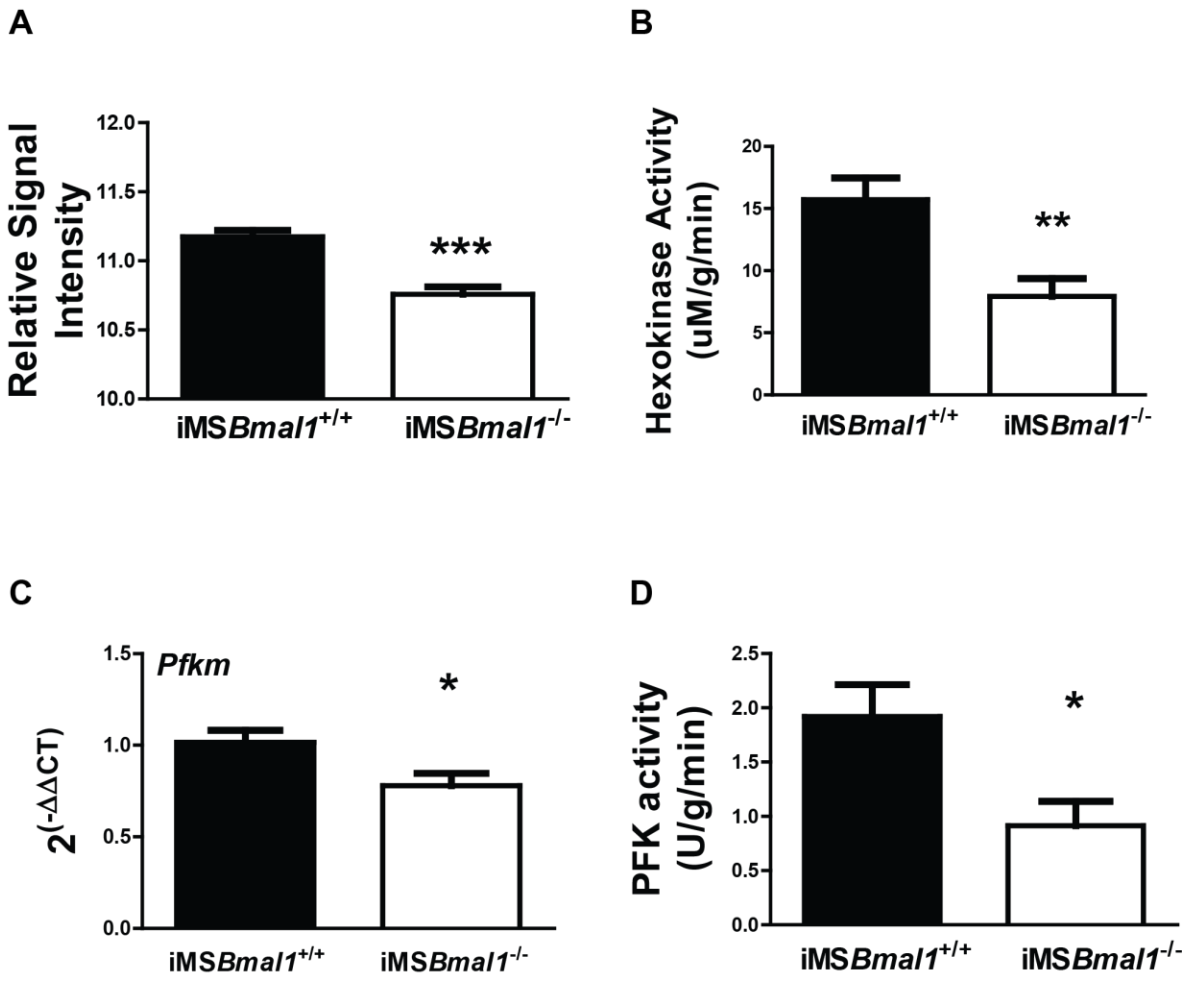


Figure 3.5. Activity of rate-limiting glycolytic enzymes (HK2 and PFK1) in the iMS*Bmal1*$^{+/+}$ and iMS*Bmal1*$^{-/-}$ mice. (A) mRNA expression of *Hk2* obtained from microarray data (n=6). (B) Enzymatic activity of the rate limiting enzyme HK2 in the gastrocnemius muscle measured using a spectrophometric assay (n=7/group). (C) mRNA expression of the rate limiting enzyme *Pfkm* in the gastrocnemius muscle (n=7/group). (D) Enzymatic activity of PFKM in gastrocnemius muscles.

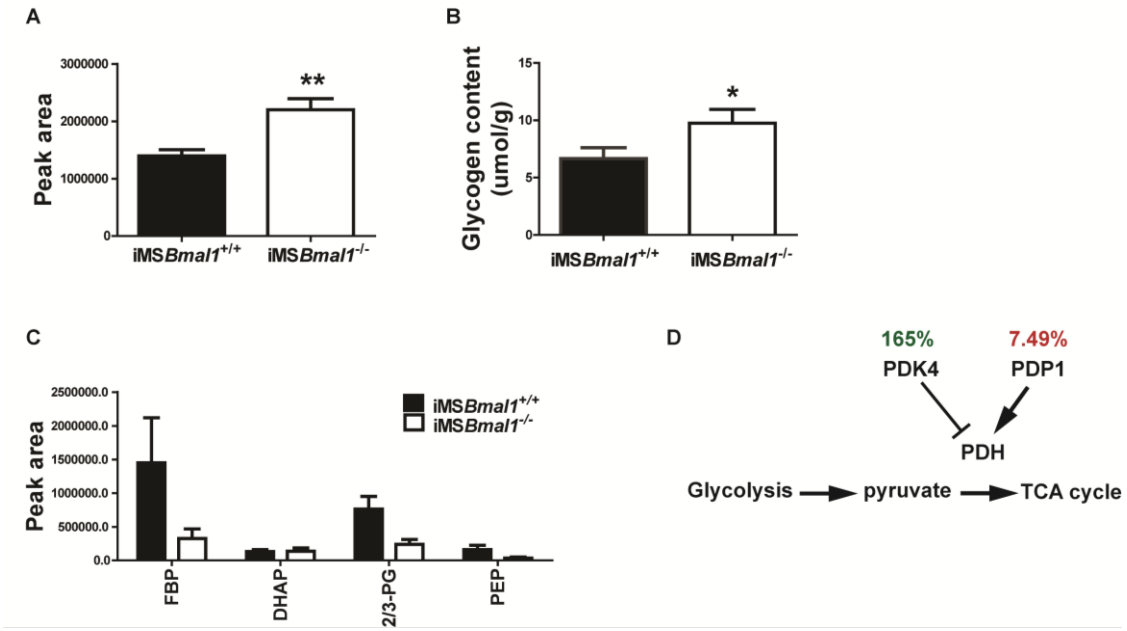


Figure 3.6. Glycolytic flux is altered in the iMS*Bmal1*$^{-/-}$ mice. (A) Metabolomics analysis of glucose in the GTN of iMS*Bmal1*$^{+/+}$ and iMS*Bmal1*$^{-/-}$ mice. Glucose (measured as peak area) is significantly increased in the skeletal musle of iMS*Bmal1*$^{-/-}$ mice (n=7/group). (B) Glycogen content in the GTN of iMS*Bmal1*$^{+/+}$ and iMS*Bmal1*$^{-/-}$ mice measured via colorimetric assay (n=32). (C) Metabolites of glycolysis from the metabolomics dataset in the iMS*Bmal1*$^{+/+}$ and iMS*Bmal1*$^{-/-}$ mice (n=7/group). (D) Schematic showing effect of Pdk4 and Pdp1 on glucose oxidation. Percentages are the change in intensity signal relative to iMSBmal1+/+ mice obtained from microarray analysis. Green depicts changes in the positive direction (increased) and red depicts changes in the negative direction (decreased).

The data presented thus far provides compelling evidence that iMS*Bmal1*$^{-/-}$ mice have altered skeletal muscle glucose metabolism. Glycolytic flux appears to be decreased, glucose uptake is significantly impaired, glucose tolerance is reduced and body composition changes in an age-dependent manner. Additional experiments were done however that resulted in confounding data. Since skeletal muscle insulin stimulated glucose uptake was significantly impaired and iMS*Bmal1*$^{-/-}$ mice were glucose intolerant, it was hypothesized that insulin sensitivity would be lower in the iMS*Bmal1*$^{-/-}$ mice. Three different doses were tested, 0.75 U/kg, 0.85U/kg and 1.0 U/kg and were referred to

as submaximal, intermediate and maximal respectively. The first dose examined was the submaximal dose. When iMS*Bmal1*$^{+/+}$ and iMS*Bmal1*$^{-/-}$ mice were injected with this dose, no significant difference was detected (**Figure 3.7A**). In contrast, when mice were given the intermediate or maximal dose, blood glucose dropped lower in the iMS*Bmal1*$^{-/-}$ mice and remained decreased for a longer amount of time (**Figure 3.7 A,B**). For the intermediate dose it is important to note that four of the eight iMS*Bmal1*$^{-/-}$ mice dropped to critical blood glucose values (below 70 mg/dL) and began to seize around 30 minutes post-injection. As a result, these mice had to be given emergency doses of glucose and were omitted from the later timepoints. As one might expect, the maximal dose had an even more severe effect and the experiment was stopped 30 minutes post-injection do to the presence of seizures in the iMS*Bmal1*$^{-/-}$ mice. These insulin tolerance tests suggest that the iMS*Bmal1*$^{-/-}$ mice are actually more insulin sensitive compared to iMS*Bmal1*$^{+/+}$ mice. Insulin sensitivity is not solely determined by skeletal muscle glucose metabolism. Other metabolic tissues such as the liver and adipose may contribute to glucose uptake and are quite important for counterregulatory responses (such as glycogenolysis, gluconeogenesis and hormone release) to insulin. In fact, liver glycogenolysis has been shown to account for approximately 90% of endogenous glucose production in healthy subjects (Kishore et al., 2006). Glycogenolysis is the breakdown of glycogen to glucose and therefore the extent to which glucose can be mobilized is dependent on glycogen content. We measured hepatic glycogen in liver tissue from the circadian time course and found that liver glycogen was significantly reduced in the iMS*Bmal1*$^{-/-}$ mice (**Figure 7D**). This may indicate that iMS*Bmal1*$^{-/-}$ mice cannot respond appropriately to insulin-induced hypoglycemia, accounting for at least some part of the significant drop in blood

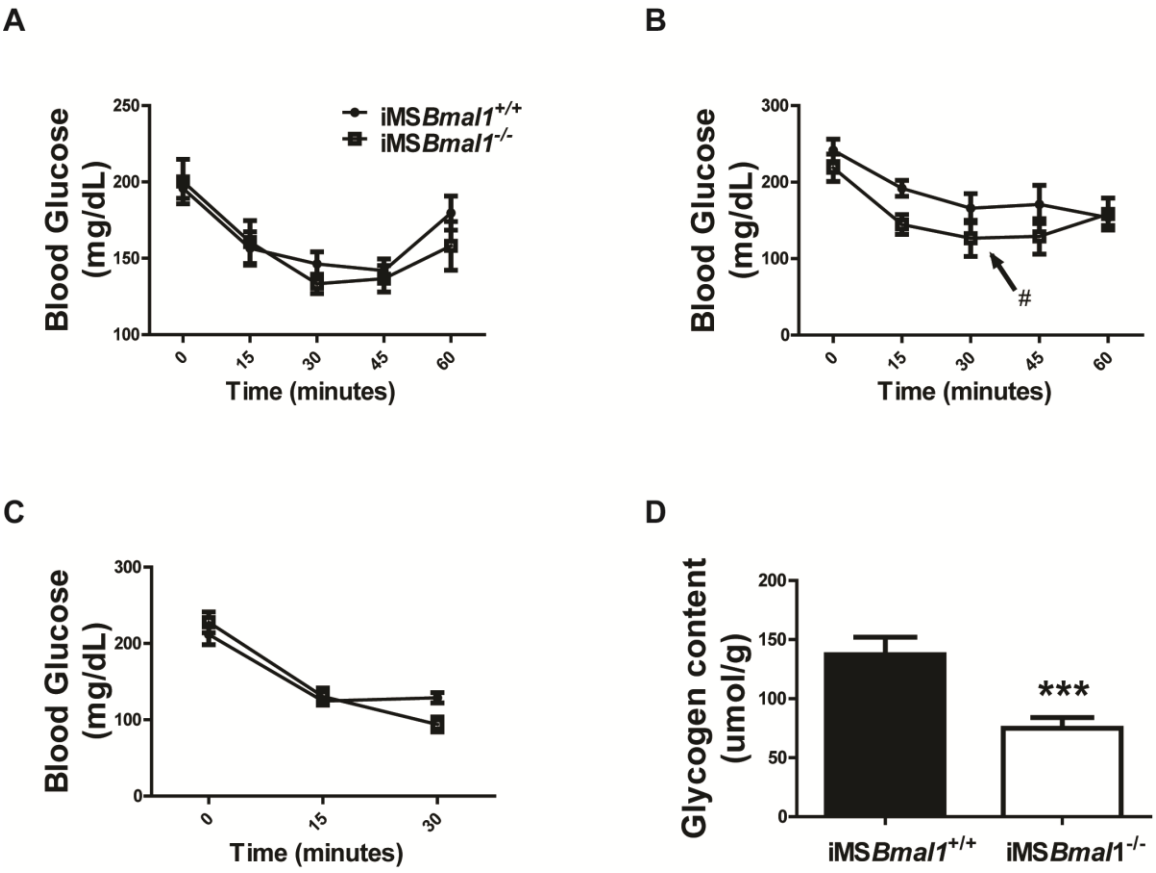


Figure 3.7. Insulin tolerance tests (n=8) and liver glycogen (n=32) in the iMS*Bmal1*$^{+/+}$ and iMS*Bmal1*$^{-/-}$ mice. (A) Insulin tolerance test using the submaxiaml dose of 0.75 U/kg. (B) Insulin tolerance test with an insulin dose of 0.85 U/kg (# indicates the point at which 4 mice were dropped from experiment due to hypglycemic seizures). (C) Insulin tolerance test using a maximal dose of 1.0 U/kg insulin. Test only goes to 30 minutes post-injection due to severe hypoglycemic symptoms in the iMS*Bmal1*$^{-/-}$ mice. (D) Liverg glycogen in the iMS*Bmal1*$^{+/+}$ and iMS*Bmal1*$^{-/-}$ mice.

glucose observed in iMS*Bmal1*$^{-/-}$ mice following intermediate and maximal insulin doses. Interestingly, this also implies that knockdown of Bmal1 exclusively in skeletal muscle has an effect on another (Bmal1 postive) tissue (liver). Further research is required in order to determine other factors that contribute to the insulin sensitivity in the iMS*Bmal1*$^{-/-}$ mouse and the mechanism by which skeletal muscle is affecting other tissues. Overall however, it is clear from the data that the inducible loss of *Bmal1* in skeletal muscle has a

substantial effect on skeletal muscle glucose metabolism. Therefore, it can be concluded that *Bmal1* and circadian rhythms have an essential role in regulating skeletal muscle glucose metabolism.

Chapter 4: Discussion

4.1. Discussion of Data

Skeletal muscle is a fundamental metabolic tissue and primarily responsible for the disposal of glucose in the postprandial state (Ferrannini et al., 1988). Circadian rhythms play an important role in regulating metabolism in a tissue specific manner and recent research demonstrates that disruption of circadian rhythms leads to metabolic dysfunction and disease (Storch et al., 2002; Schmutz et al., 2012; Dyar et al., 2014; Hodge et al., 2015b). Our understanding of the function of the clock and core clock genes, such as *Bmal1*, in skeletal muscle is still quite elementary. In our investigation we demonstrated that loss of the circadian core clock gene, *Bmal1*, in exclusively skeletal muscle results in: impaired glucose uptake with concomitant reduction in the muscle glucose transporter GLUT4, decreased glycolytic flux with a potential shift in substrate utilization, hyperglycemia in the non-fasting condition, glucose intolerance and changes in overall body composition.

Dyar et. al. previously showed that loss of skeletal muscle *Bmal1* resulted in impaired insulin-stimulated glucose uptake (Dyar et al., 2014). We expanded on this by showing that not only is insulin-stimulated glucose uptake impaired, but AICAR-stimulated glucose uptake is diminished as well. AICAR signals through the same pathway as contraction, which is distinct from insulin signaling (Brozinick et al., 1992; Sakamoto and Goodyear, 2002). The pathways do have some common components, one of which is the glucose transporter GLUT4. Both insulin- and AICAR- stimulated signaling result in the translocation of GLUT4 from intracellular vesicles to the plasma

membrane where it can transport glucose into the cell. We found that *Glut4* mRNA expression was significantly decreased in the iMS*Bmal1*$^{-/-}$ mice. We also observed a significant reduction in GLUT4 protein content which is consistent with GLUT4 protein data in the Dyar et. al. study (Dyar et al., 2014).

Glucose uptake can also be affected by HK2 activity. HK2 phosphorylates glucose when it enters the cell thereby maintaining a concentration gradient so that glucose may passively enter the cell by facilitated diffusion via GLUT4 (Osawa et al., 1995; Osawa et al., 1996; Fueger et al., 2003; Wasserman et al., 2011; Richter and Hargreaves, 2013). We observed as decrease in *Hk2* expression in our microarray data which is consistent with the decrease in HK2 protein content reported by Dyar and colleagues (Dyar et al., 2014). More notably, we measured enzymatic activity and observed a significant reduction in HK2 activity in iMS*Bmal1*$^{-/-}$ mice. This is important to point out because although HK2 protein content has been previously reported, total HK2 enzymatic activity was unknown. The decrease in HK2 activity likely contributes to the decrease in glucose transport. In fact, a study by Fueger et. al. in 1995 demonstrated that partial *Hk2* knockdown was enough to result in a decrease in an impairment in glucose uptake (Fueger et al., 2003).

HK2 is a key enzyme of the glycolytic pathway and therefore crucial for carbohydrate metabolism. A previous publication from our lab evaluating microarray data suggests that one of the key functions affected by loss of skeletal muscle *Bmal1* is carbohydrate metabolism. The study shows that a number of genes important for carbohydrate metabolism display decreased expression (Hodge et al., 2015b). The HK2 data from this dissertation provides initial data supporting this idea. We explored this

further by looking at the other critical glycolytic rate-limiting enzyme PFK1 (Crabtree and Newsholme, 1972; Nakajima et al., 2002). Both *Pfk1* mRNA expression and PFK1 enzyme activity were significantly reduced in the iMS*Bmal1*$^{-/-}$ mice. Taken together, the decrease in expression and dampening of activity of these two rate-limiting enzymes presents strong evidence for impaired glucose catabolism and glycolytic flux.

Metabolomics data was obtained for the gastrocnemius muscle of iMS*Bmal1*$^{+/+}$ and iMS*Bmal1*$^{-/-}$ mice and provided additional data on changes in skeletal muscle metabolism. In comparing peak areas from the metabolomics data set, we observed further evidence of reduced glycolytic flux. Muscle glucose content was significantly higher, while many glycolytic metabolites showed a trend toward or significant decreases in the iMS*Bmal1*$^{-/-}$ mice relative to the iMS*Bmal1*$^{+/+}$ mice. Glyceraldehyde 3-phosphate and phosphoenolpyruvate trended lower while 2-phosphoglycerate and 3-phosphoglycerate were significantly decreased. To add to our understanding of glucose handling we conducted colorimetric assays to measure glycogen content and showed significantly increased muscle glycogen. These data suggest that glucose is not being sufficiently utilized in skeletal muscle and is instead being stored.

In light of the fact that skeletal muscle, a vital metabolic tissue, cannot efficiently dispose of glucose and utilize it as a fuel source, one can expect that this will affect overall metabolic health. Skeletal muscle is a critical depot for glucose in the postprandial state, so with impaired glucose uptake it is logical that non-fasting blood glucose would be elevated. The glucose being consumed would normally trigger insulin release which would act on tissues (mainly skeletal muscle) to stimulate glucose uptake, but this process is defective in the iMS*Bmal1*$^{-/-}$ mice. Fasting glucose is not changed in our mice

but this is possibly because in the fasting state the mice have time to clear the glucose through uptake into other metabolic tissues. Or perhaps a small amount of glucose is taken up into skeletal muscle but at a much slower rate. The impairment in glucose uptake and oxidation also helps explain the glucose intolerance in the iMS*Bmal1*$^{-/-}$ mice. In a normal glucose tolerance test one would expect the exogenous bolus of glucose to cause insulin release resulting in increased glucose uptake. Since iMS*Bmal1*$^{-/-}$ mice have impaired skeletal muscle insulin-stimulated glucose uptake, they do not respond appropriately to the bolus of glucose. Instead blood glucose goes up much higher and takes a longer amount of time to be cleared.

The metabolic phenotype observed in the iMS*Bmal1*$^{-/-}$ mice is congruent with results reported in past literature using partial or full knockout models of *Glut4*, *Hk2* and *Pfk1*. A 2000 study examining the effect of muscle specific *Glut4* knockout showed that the mice gained weight more slowly and had reduced fat stores (Zisman et al., 2000). This is consistent with the decrease in body fat composition observed in our mice. This same study and some studies on hexokinase- and phosphofructokinase-deficient mice demonstrate development of insulin resistance, glucose intolerance and elevated blood insulin (Zisman et al., 2000; Nakajima et al., 2002; Fueger et al., 2003). Loss of *Bmal1* in the skeletal muscle of our mice led to deficiencies in the expression and/or activity of *Glut4*, *Hk2* and *Pfk1* and as such, the observed glucose intolerance, increased non-fasting glucose and elevated insulin are in agreement with past studies. One interesting fact to note is that where we saw glucose intolerance following skeletal muscle specific *Bmal1* knockout, the Dyar study did not. However, this could be due to differences in the ages at which mice were injected with tamoxifen. We waited to inject our mice with tamoxifen

until 12 weeks of age. This was done to avoid developmental factors. During development, satellite cells (which are normally quiescent) may fuse into muscle to contribute to growth. The inducible model used by our lab and the Dyar study does not knock down *Bmal1* in satellite cells. Therefore, if satellite cells fuse in it results it can result in partial rescue of *Bmal1*. The Dyar study injected their mice at an earlier age and therefore the mice may have had satellite cell fusion and only be partial knockouts. This may account for the differences reported for glucose tolerance. It is also important to point out that while the Dyar paper published data demonstrating impaired insulin-stimulated glucose uptake in their inducible skeletal muscle *Bmal1* knockout model, the rest of the data in this dissertation is novel. Dyar and colleagues did not investigate other methods of glucose stimulation. Furthermore the GLUT4, HK2, blood glucose and metabolomics data presented by Dyar was not done in the inducible knockout model. Therefore, although many of findings in dissertation were consistent with findings in the muscle specific knockout mice Dyar used, they are important findings because they demonstrate that changes in GLUT4, HK2, blood glucose and metabolomics are not to developmental effects of skeletal muscle *Bmal1* deficiency (Dyar et al., 2014).

It is quite clear that glucose uptake and glucose oxidation in skeletal muscle is impaired in the iMS*Bmal1*$^{-/-}$ mice. Based on these findings it was expected that insulin sensitivity would be reduced as well. Remarkably, when iMS*Bmal1*$^{-/-}$ mice were tested with the higher doses of insulin, they showed improved sensitivity to insulin. Blood glucose levels in the iMS*Bmal1*$^{-/-}$ mice dropped lower and remained lower for a longer period of time relative to iMS*Bmal1*$^{+/+}$ mice. Some iMS*Bmal1*$^{-/-}$ mice even went into seizures and required a bolus of glucose. This was completely unexpected since the

skeletal muscle presented as insulin resistant. There are two things in the insulin response curve that be addressed. First is the fact that blood glucose dropped lower at higher doses of insulin in the iMS*Bmal1*$^{-/-}$ mice relative to iMS*Bmal1*$^{+/+}$ mice. Since insulin-stimulated uptake is impaired in the iMS*Bmal1*$^{-/-}$ mice, one possible explanation is that other metabolic tissues, such as the liver and adipose tissue, are compensating and have increased insulin sensitivity or glucose uptake. The second part of the response curve that requires some consideration is the delayed recovery of normal blood glucose levels in the iMS*Bmal1*$^{-/-}$ mice. When blood glucose drops to low levels, a number of things generally happen to negatively regulate glucose levels. As glucose levels begin to drop, pancreatic beta cell release of insulin decreases and glucagon release from the alpha cells increases. As blood glucose drops below 70 mg/dL, the adrenal medulla is stimulated to secrete the catecholamines epinephrine and norepinephrine. Two other hormones released are growth hormone and cortisol. Glucagon, epinephrine, norepinephrine, growth hormone and cortisol have anti-insulin effects and work to increase blood glucose levels. They stimulate gluconeogenesis and glycogenolysis thereby generating and mobilizing glucose (Sprague and Arbelaez, 2011; Marieb and Hoehn, 2012). Liver glycogenolysis is responsible for approximately 90% of endogenous glucose production in healthy subjects (Kishore et al., 2006). Our liver glycogen assay showed that liver glycogen stores were significantly decreased. With reduced liver glycogen, the ability of the liver to deliver glucose may be diminished. Interestingly, Kishore and colleagues have demonstrated that intensively treated diabetics may present with defective counterregulation due to inability of the liver to increase glycogenolysis in response to hypoglycemia. Although are mice are not intensively treated diabetics, they do present with significantly increased blood

insulin which might be considered similar to intensive insulin treatment in diabetic patients (Kishore et al., 2006).

Despite unexpected results for insulin tolerance assays, this investigation reveals some exciting new findings. Studies evaluating the role of the circadian clock and clock genes in skeletal muscle metabolism are limited. Past research has shown that insulin-stimulated glucose uptake is impaired and GLUT4 protein and HK2 protein are down (Dyar et al., 2014). The data in this dissertation confirms those finding but goes even further in elucidating *Bmal1* function in skeletal muscle metabolism. The current study demonstrates that not only is insulin-stimulated glucose uptake impaired but AICAR-stimulated uptake is defective as well. These two types of glucose uptake signal through different pathways but share a common endpoint of GLUT4. We confirmed that GLUT4 protein content was reduced and showed that mRNA expression was also reduced. We showed that *Hk2* expression was decreased and went a step further than the Dyar study and demonstrated that HK2 enzyme activity was reduced (Dyar et al., 2014). Our data exhibits that the other rate limiting glycolytic enzyme, PFK1, has reduced enzyme and reduced mRNA expression. This suggests glycolytic activity is down and we showed that glycolytic metabolites are lower. Glycogen however was increased indicating glucose is stored in skeletal muscle in lieu of being oxidized. Based on these results at the level of the skeletal muscle we expected changes in overall metabolic health. In contrast to the Dyar paper, we presented evidence of glucose intolerance in skeletal muscle *Bmal1* knockout mice (Dyar et al., 2014). Our experiments produced data displaying increased non-fasting glucose and elevated fasting plasma insulin. Furthermore, by 12 week post-recombination we observed significant changes in body composition. Taken together

these data reveal a crucial role for skeletal muscle *Bmal1* in the regulation of skeletal muscle metabolism and overall metabolic health. Additionally, the insulin tolerance and more notably the liver glycogen results imply that skeletal muscle *Bmal1* and metabolism may affect the metabolic function of other tissues such as the liver. This is exciting novel data that advances our current knowledge of skeletal muscle circadian rhythms (especially core clock gene *Bmal1*) and metabolism.

4.2 Future experiments

Although this study is important in highlighting a novel role for *Bmal1* in regulating insulin- and AICAR-stimulated glucose uptake, glucose oxidation and metabolic health, there is still much that is unknown. Further research is required to elucidate the exact mechanism by which *Bmal1* alters GLUT4 content, glucose uptake, and glycolytic gene expression and activity in skeletal muscle. There are a variety of metabolic transcription factors that present with circadian expression patterns so it may be of value to start by evaluating the impact of *Bmal1* knockdown on expression of these transcription factors. For instance, *Sp1* and the PPARs have been shown to regulate Glut4 transcription (Marin-Juez et al., 2013). *Sp1* and *Ppard* have e-box sequences that could be bound by CLOCK:BMAL. In addition, *Sp1* and *Pgc1a* (a coactivator for the PPARs) have been demonstrated to be regulated in some part by circadian rhythms (Mendez-Ferrer et al., 2008; Miyazaki et al., 2011). Besides regulating *Glut4* expression, *Sp1* and *Ppard* have also been shown to regulated *Hk2* transcription (Archer, 2011; Panasyuk et

al., 2012). In order to uncover the mechanism by which loss of Bmal1 results in downregulation of *Glut4* and *Hk2,* impaired glucose uptake and decreased glycolytic flux, it would be beneficial to assess *Sp1* and *Ppard* in the iMS*Bmal1*$^{-/-}$ mice. One might also conduct luciferase assays in order to evaluate overexpression and knockdown of *Bmal1* in skeletal muscle cells on *Sp1* and *Ppard* expression. If loss of *Bmal1* results in decreased *Sp1* and *Ppard* expression it could partially explain the reduced *Glut4* and *Hk2* and begin to get at a mechanism.

Another substantial question emerging from this dissertation is the question of how insulin tolerance appears to be improved in the iMS*Bmal1*$^{-/-}$ mice. As mentioned previously, other tissues metabolic tissues may be compensating. The gold standard for evaluating physiological responses to insulin is clamp studies. To further examine insulin action on blood glucose and obtain data on glucose disposal and output, clamp studies would be required. To go even deeper into uptake by different tissues a study could be done using clamp studies and radiolabeled glucose (Chen et al., 2006; Tam et al., 2012). With this, it would be possible to determine the contribution of the different tissues to glucose clearance. The iMS*Bmal1*$^{-/-}$ mice not only exhibited a greater drop in blood glucose in response to insulin, but they took longer to return to normal. This implies a defect in counterregulatory mechanisms. As previously mentioned, counterregulatory mechanisms involve release of hormones (glucagon, epinephrine, norepinephrine, growth hormone and cortisol, increased gluconeogenesis and increased glycogenolysis (Sprague and Arbelaez, 2011). Therefore, it would be informative to collect blood during an insulin tolerance test and test for levels of counterregulatory hormones. In addition, one might

look at major gluconeogenic and glycogenolytic proteins in the liver, which is responsible for the majority of endogenous glucose production (Kishore et al., 2006).

The current dissertation only went out to 12 weeks post-recombination in the iMS*Bmal1*$^{-/-}$ mice. For the most part experiments were done only at the 5 week post-recombination timepoint. Another possible study would be to follow these metabolic phenotype of these mice to later timepoints. The body composition may change even more and the impairments in glucose uptake and glycolytic flux may become even more severe. Our mice exhibited significantly elevated plasma insulin at 5 weeks post-recombination but perhaps they will lose insulin secretion all together with aging. This would be similar to what is observed in type II diabetes. Initially insulin secretion is higher to compensate for the insulin resistance, but this causes inflammation and damage to the pancreatic beta cells eventually resulting in loss impaired insulin secretion (Marieb and Hoehn, 2012).

A very intriguing experiment would be experiments involving changes in diet. The iMS*Bmal1*$^{-/-}$ mice appear to have decreased glucose oxidation. It would be very interesting to put these mice on mostly-carbohydrate (low-fat), or all-carbohydrate diets and observe the effects. Metabolic cage experiments could be done to obtain data such as activity, feeding, RER and VO_2. Exercise experiments (run-to-exhaustion) could be done on normal and altered diets to see how iMS*Bmal1*$^{-/-}$ mice perform. Overall health could be observed over time. Since the iMS*Bmal1*$^{-/-}$ mice don't oxidize glucose well, it would be expected that mice would not perform well on an all carbohydrate diet and may get sick and show symptoms much more quickly.

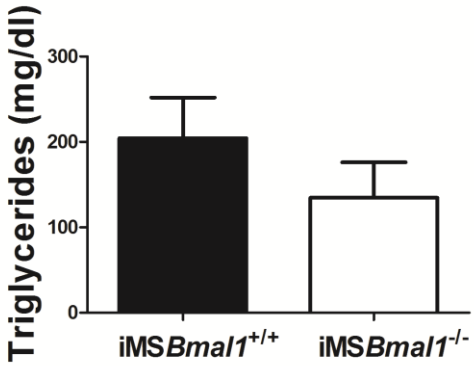
Finally, it would be interesting to do experiments in which Bmal1was rescued in the iMS*Bmal1*$^{-/-}$ mice to see if the skeletal muscle glucose uptake and glycolysis could be improved. Perhaps one could damage the muscle on one side of the body using BaCl in order to stimulate satellite cell fusion in that muscle. As mentioned earlier, satellite cell *Bmal1* is not knocked down in our model, so if they fuse into the muscle they add in *Bmal1* (Fry et al., 2014). Another method would be to transfect *Bmal1in vivo* back into the muscle (Wan et al., 2012). Then experiments on the skeletal muscle (glucose uptake, glycogen assays, metabolomics) could be repeated to evaluate if any improvement occurred.

4.3 Future Goals/Plans

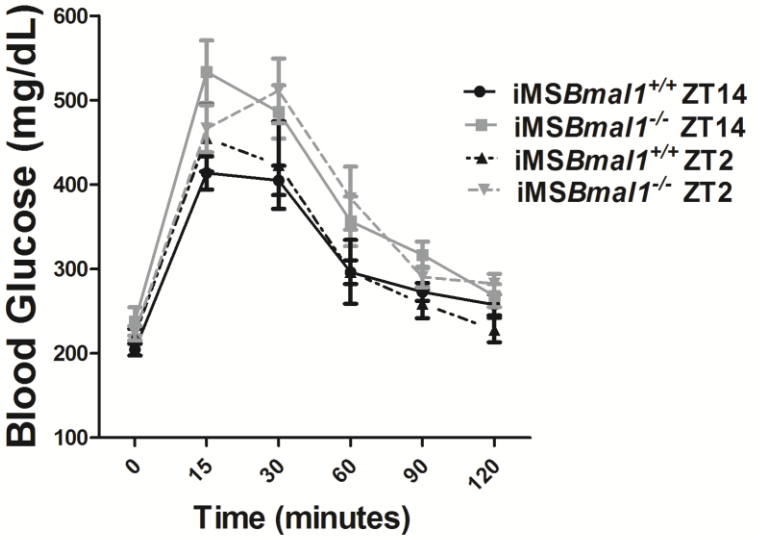
My immediate plans after graduation involve applying for scientific policymaking fellowships (AAAS, presidential management, etc.). Most of the fellowship applications are due this fall, finalize decisions in next spring and begin next fall. My primary goal is to get one of these fellowships so that I could learn and gain experience in the 2016-17 year and try to work somewhere such as NIH, NSF or the FDA in establishing policy or as a grants officer. I also have a strong interest in teaching, in particular at a liberal arts type college/university. I started teaching at Bluegrass Community and Technical College this last summer and and going to continue teaching there. I am also getting applications together for Eastern Kentucky University, Asbury University and Transylvania University for adjunct faculty positions. I want to gain as much teaching experience as possible while I wait to hear about the scientific policymaking fellowships. This will

allow me to build my CV while I wait and then if I am not fortunate enough to get a fellowship I have instructing experience and will pursue a full-time job teaching. I have been lucky enough to have science professors along the way that have made learning exciting for me and inspired me. Hopefully I will be able to do that for students I may have in the future.

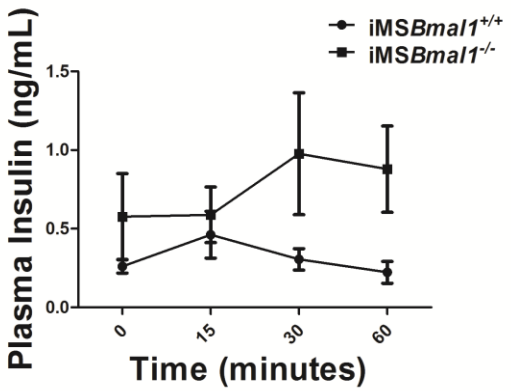
APPENDIX:



Plasma triglycerides in the iMS*Bmal1*$^{+/+}$ and iMS*Bmal1*$^{-/-}$ mice. Blood was collected 5 weeks post-recombination, 2 hours after the lights went off (n=7/group; 2 mice in each group were female).



Glucose tolerance at ZT2 (n=7 iMS*Bmal1*$^{+/+}$ and 10 iMS*Bmal1*$^{-/-}$; 3 tamoxifen mice were female) and ZT14 (n=8/group; 2 mice in each group were female) in the iMS*Bmal1*$^{+/+}$ and iMS*Bmal1*$^{-/-}$ mice. To differentiate between genotype, black lines were used for iMS*Bmal1*$^{+/+}$ mice and gray lines were used for iMS*Bmal1*$^{-/-}$ mice. To differentiate between time of test, dotted lines were used for ZT2 while soid lines were used for ZT14.



Plasma insulin during a glucose tolerance test in the iMS*Bmal1*$^{+/+}$ and iMS*Bmal1*$^{-/-}$ mice. Baseline insulin was measured and then mice were injected (IP) with a bolus of glucose (2g/kg body weight). Blood was collected at 15, 30 and 60 minutes post glucose injection (n=7/group; 2 female mice per group).

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VITA

Brianna D. Harfmann

Education

2007-2011 Bachelors of Science (Exercise and Health Science), Alma College, Alma, MI

Professional Positions

2011-2015 Doctoral Candidate, University of Kentucky, College of Medicine, Lexington, KY
Advisor: Karyn A. Esser, Ph.D.
Defense Date: October 7, 2015

2015 Adjunct Instructor, Bluegrass Community and Technical College - Biology Department, Lexington, KY

Honors

2014-2015 Graduate Student Representative – UK Department of Physiology
2014 Nominee, University of Kentucky Presidential Graduate Fellowship
2013-2014 SAHA Cardiovascular trainee, T321T32 HL072743, Training Grant
2013 International Chronobiology Summer School attendee
2011 Graduate School Academic Year Fellowship- University of Kentucky
2011 Summa Cum Laude- Alma College
2011 Finalist for Alma College Barlow Trophy
2010-2011 Omicron Delta Kappa Honor Society- Alma chapter
2009-2011 Exercise and Health Science Honor Society (President 2010-2011)
2007-2011 Harold A. Baker Leadership Scholarship– Alma College
2007-2011 Dean's List– Alma College
2007-2009 MIAA Academic Honor Roll

Peer-reviewed Publications

Schroder EA, **Harfmann BD**, Zhang X, Srikuea R, England JH, Hodge BA, Wen Y, Riley LA, Yu Qi, Christy AD, Smith JD, Wolf Horrell EM, Mula J, Peterson CA, Butterfield TA and Esser KA. Intrinsic muscle clock is necessary for musculoskeletal health. J Physiol, 2015.

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Harfmann BD, Esser KA. 2013. Loss of skeletal muscle circadian rhythms leads to altered metabolism. *Oral Presentation – Gill Heart Institute Cardiovascular Research Day, Lexington, Kentucky*

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Harfmann BD, Srikuea R, Esser KA. 2012. Loss of *Bmal1* leads to accelerated aging of skeletal muscle. *Poster Presentation – Center for Muscle Biology, Lexington, Kentucky*

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